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14. ABSTRACT During the first two year of the award, we made several high impact key observations: (I) Prophylactic treatment with Farnesylthiosalicylic acid (FTS), a first-in-class oral selective RAS inhibitor, provides a significant immunomodulatory effect in the rat adjuvant-induced arthritis (AIA) model by all outcome parameters (Clinical assessment and relevant laboratory/immunological/Molecular analyses). (II) Prophylactic dosing of FTS combined with methotrexate (MTX) provides a synergistic protective effect (~90% disease inhibition). (III) The FTS derivative, F-FTS, showed superior therapeutic clinical efficacy compared to FTS in the AIA model. (IV) The detailed bioinformatics analysis of the transcriptomes of relevant splenic CD4+ T cells show that FTS/F-FTS are potent suppressors of the in vivo induction of a TH17 immune response. (V) FTS had a non-inferior therapeutic effect as compared to MTX in the collagen type-II induced arthritis (CIA) mouse model. (VI) FTS semi-prophylactic treatment alone or combined with MTX was coupled with significant "positive" attenuation of multiple relevant immunological and laboratory markers - all strongly implying that the main biological effect of FTS/F-FTS is inhibition of a pathogenic TH17 autoimmune response to collagen II.					
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Ras-GTPases are molecular switches that regulate key cellular processes, such as proliferation, differentiation, apoptosis, and motility. In T cells, Ras-family GTPases (e.g. K/N-Ras) are crucial for proper TCR-dependent activation following antigen recognition. Defective Ras GTPases signaling has been associated with T cell anergy, and accordingly increased expression of active Ras was shown to reverse anergy and to restore IL-2 production. Importantly, T cells from patients with Rheumatoid Arthritis (RA) display augmented activation of the Ras/Raf/MEK/ERK1/2 signaling pathway, and accordingly overexpression of active K-RAS in normal CD4⁺ T cells has been shown to promote T cells reactivity to relevant autoantigen in RA. Thus, Ras GTPases appear to be a promising molecular target for inhibiting T cell activation in RA. Based on an innovative concept Kloog (the partnering PI) and colleagues discovered a potent non-toxic inhibitor of Ras, Farnesylthiosalicylic acid (FTS). This small molecule does not belong to the class of farnesyltransferase inhibitors (FTIs) that failed in clinical trials. It interferes with the interactions between Ras and distinct prenyl-binding chaperone proteins that are vital for the proper plasma membrane (PM) localization and signaling dynamics of Ras-GTPases, and indeed FTS dislodges the classical H/N/K-Ras GTPases from the PM and inhibits their effective downstream signaling. In multiple preclinical animal studies it has been shown that FTS effectively inhibited *in vivo* tumor growth of oncogenic K/N-Ras-dependent cancers. Thus, in collaboration with Concordia Pharmaceuticals Inc., FTS was developed into an oral drug, Salirasib®. The drug has been already tested in the clinic for the treatment of cancers with oncogenic mutations in *KRAS* and *NRAS*. No dose-limiting toxicities or major adverse events were reported during Salirasib® treatment, in phase I clinical trials of patients with advanced pancreatic cancer, hematological malignancies (NCT00867230; M.D. Anderson Cancer Center, TX), and in phase II clinical study in non-small-cell lung cancer patients (NCT00531401; Memorial Sloan Kettering Cancer Center, NY). Thus, Salirasib® is the only available successful Ras GTPases inhibitor that reached Phase II clinical trials, and moreover received an orphan drug designation by the FDA for the treatment of pancreatic cancer. Importantly, we have extensively studied the effects of FTS and related derivatives (e.g., FTS-Amide and FTS-methoxymethylester), in multiple pre-clinical animal models of autoimmune

inflammation, such as: experimental autoimmune encephalomyelitis; Type-1 diabetes; colitis and others. More recently, our preliminary studies in the adjuvant-induced arthritis (AIA) rat model – a classical animal model for RA – imply that FTS attenuates disease manifestation, as assessed by: clinical scores; MRI imaging; histopathology; and serum levels of pro-inflammatory cytokines. Thus, our working hypothesis is that Salirasib® has a good potential to be a “silver bullet” drug for RA and other T cell-dependent autoimmune disorders.

Our objectives are to test further this hypothesis in the AIA model as well as in another established animal model of RA, the collagen type-II induced arthritis (CIA) in DBA/1 (H-2q) mice. In parallel we will study *in vitro*, the effects of FTS and its different newer derivatives on a wide range of T cell functions and signaling networks implicated in RA. For the therapeutic treatment protocol, we will be administer FTS orally by gavage, once daily, starting immediately after disease initiation. Multiple modalities will be used to assess joint inflammation/damage and the immune response, as follows: arthritis clinical scores; MRI scans, micro-CT; histopathology examination by a blinded pathologist; serum cytokine profiles; T cell subset analysis (e.g., Foxp3+ Treg, Th1, Th17, etc.) by polychromatic flow cytometry. Additionally, we will analyzed the activation of different Ras downstream effectors and relevant cellular programs by Western blotting, Affymetrix GeneChip® whole-transcript arrays, and quantitative real-time PCR analysis. The proposed project is highly relevant to the FY13 PRMRP topic area of Rheumatoid Arthritis (RA). The short-term impact of our research will be an improved understanding of the role of Ras GTPases in shaping, tuning, and regulating the autoimmune T cell response, and the effects of Ras inhibitors on the pathogenesis of the inflammatory arthritis in two relevant pre-clinical models of RA. We envision that the long-term impact of our proposed research plan will be the introduction of a new class of synthetic drugs, orally available small molecule Ras-inhibitors such as Salirasib®, to advance the clinical management of RA patients with conceivably fewer side effects and reduced healthcare system costs compared to biologic drugs.

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Ras GTPases are molecular switches that regulate key cellular processes and in T cells they are necessary for proper TCR-dependent activation following antigen recognition. Reduced Ras signaling has been associated with T cell anergy and defective IL-2 production (1, 2). Importantly, synovial T cells from patients with RA display augmented activation of the Ras/Raf/ ERK pathway (3, 4). Thus, Ras GTPases appear to be a promising molecular target for inhibiting T cell activation in RA. Based on an innovative concept Klooog (the partnering PI) and colleagues discovered a potent non-toxic inhibitor of Ras, Farnesylthiosalicylic acid/FTS (5, 6). In collaboration with Concordia Pharmaceuticals Inc., FTS was developed into an oral drug, Salirasib®. The drug was already tested in the clinic for the treatment of cancers with oncogenic mutations in *KRAS* and *NRAS*. No dose-limiting toxicities or major adverse events were reported during Salirasib® treatment, in phase I clinical trials of patients with advanced solid cancer. Thus, Salirasib® is the only available successful Ras GTPase inhibitor that reached clinical trials, which moreover received an orphan drug designation by the FDA for the treatment of pancreatic cancer (7, 8). In the first and second years of this project, our aim was to complete the following tasks: (i) Test in the rat AIA model of RA (rheumatoid arthritis) the prophylactic and therapeutic efficacy of FTS on relevant clinical outcomes and immunological parameters; (ii) Validate in CIA mouse model the prophylactic/therapeutic effects of FTS and FTS derivatives; and (iii) test the effect of FTS as an add-on therapy to MTX in the AIA rat model.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Ras GTPases; Rheumatoid Arthritis (RA); Farnesylthiosalicylic acid (FTS); Adjuvant-induced Arthritis (AIA); T cells; T-helper cells, T regulatory cells (Treg), disease-modifying antirheumatic drugs (DMARDs); targeted synthetic DMARDs

3. **OVERALL (First & Second Year) PROJECT SUMMARY:**

In **Major Task 1** of the revised SOW, we proposed to test in the Lewis rat Adjuvant induced arthritis (AIA) model of Rheumatoid Arthritis (RA) the prophylactic and therapeutic effects of FTS.

Subtask 1A: Analyze the prophylactic/therapeutic effects of FTS on clinical AIA progression and joint pathology (**months 1-15**).

As detailed in the Annual Technical reporting of the first year, we have already studied broadly the clinical effects of prophylactic dosing of FTS and its derivative F-FTS on the progression of AIA in Lewis rats as compared to vehicle control treatment. As a positive control, we treated the rats with methotrexate (MTX) that is an effective immunosuppressant widely used in the treatment of RA patients.

Adjuvant induced arthritis in Lewis rats is an experimental model of polyarthritis that has been extensively employed for preclinical testing of numerous anti-arthritic agents, including drugs that are currently being tested in clinical trials or are currently used as novel therapeutics in RA (9). The arthritis is induced by injection of an arthrogenic preparation of complete Freund's adjuvant (CFA), prepared by suspending powdered heat-killed *Mycobacterium tuberculosis* in mineral oil at 10 mg/ml. The hallmark of this model is consistent onset and progression of robust and easy to measure polyarticular inflammation associated with marked tissue/synovial inflammation and subsequent articular bone resorption. In our hands, in agreement with previous works, clinically evident arthritis of the ankle joint usually developed ~10 days post CFA injection that progressed, in untreated control animals, into severe poly-arthritis within a few days.

We employed two dosing models:

- Prophylactic – Begin on day +1 and continue until the end of experiment.
- Therapeutic – Begin on disease onset (day +9) until study termination.

Clinical Assessment:

To assess disease progression, both clinical scoring (0-16 scale) caliper measurements of ankle joint width were done once prior to the onset of arthritis, and subsequently every other day until the study was terminated.

Histopathological Assessment:

At termination, the tibiotarsal joint was transected at the level of the medial and lateral malleolus for Histopathological Assessment. Ankle joints were then collected into 10% paraformaldehyde, for at least 24 hours, and then placed in a decalcifier solution. When decalcification was completed, the ankle joint was transected in the longitudinal plane and joints were processed for paraffin embedding, sectioned and stained with hematoxylin & eosin. Arthritic ankles were then given scores on a scale of 0-5 for inflammation and bone resorption by an experienced pathologist blinded to the animal treatment protocol, as previously described (10).

- i. As detailed in the first year report, to determine whether prophylactic dosing with FTS suppresses the clinical signs of AIA, we started daily treatment of rats from day +1 post CFA immunization, in the experimental arm, with oral FTS (100 mg/kg). Control rats received oral solution of 0.5% carboxy methyl cellulose (CMC vehicle). As a "positive control", we treated a group of Lewis rats with weekly *i.p* injection of MTX (0.5 mg/kg). AIA progression and severity was scored using a clinical index of 0 to 16 (0-4 scale for each paw). In parallel, we also assessed disease progression by a more "objective" method, using caliper measurements of ankle joint width prior to the onset of arthritis, and then every other day until the study termination on day +21 post CFA injection. As we have already determined in the first year reporting that the arthritis scores and ankle diameters were significantly reduced in the FTS treatment group compared to CMC vehicle treated rats. Importantly, we also found that the magnitude of effect of FTS and MTX on these clinical outcomes was rather similar.

- ii. In the first year, we reported that histopathological assessment of Ankles of arthritic CMC vehicle treated rats showed extensive infiltration of joints tissue with mononuclear cells (inflammation scores ranging from 4 to 5, n=8), and significant bone destruction (bone resorption scores ranging from 4 to 5). In contrast, the histopathological assessment of joint sections from FTS-treated rats showed significant reduction in joint inflammation (inflammation scores ranging from 2 to 3, n=8), and significantly less destruction of trabecular and cortical bone in the distal tibia (bone resorption scores ranging from 2 to 3).

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- iii. In this reporting period, we also performed immunohistochemistry assessment of deparaffinized and rehydrated arthritic rat ankle joint sections for the infiltrations of T lymphocyte into the synovial tissue, as detected using anti-CD3 antibodies. As shown in Figure 1, the arthritic joints of CMC vehicle treated rats showed extensive infiltration of the synovial tissue with CD3⁺ T cells, which was significantly reduced in the joints of FTS treated mice (data not shown). This implies that FTS and its derivative target the T cell response and subsequent homing into target synovial tissues.

Responsible PI: Itamar Goldstein, Tel Aviv University.

- iv. In accordance with our Institutional Animal Care and Use Committee (IACUC) guidelines, the PIs are required to ensure that the 3Rs principles are implemented. Thus, to comply with the principle of reduction, the partnering PIs have agreed to combine some experiments (subtasks) related to task 3 at this stage. Because, we already had a MTX and FTS treatment groups in Task 1 related experiments, we could easily test the efficacy of FTS as an add-on therapy to MTX during this report period and ahead of schedule. The results of these experiments were outstanding, as we found that the combined treatment of FTS + MTX almost completely inhibited the development of clinically evident arthritis as well as ankle joint swelling by caliper measurements (Figure 1A&B). Moreover the tissue sections from FTS+ MTX treated rats showed only mild joint tissue infiltration with mononuclear cells (inflammation scores ranging from 2 to 3) and only rare areas of trabecular or cortical bone resorption not readily apparent on low magnification (average bone resorption scores of 2). In comparison, as detailed above, the ankle joint tissue sections from CMC vehicle treated rats, generally showed extensive infiltration with mononuclear cells and bone destruction.

Responsible PI: Itamar Goldstein, Tel Aviv University

- v. The FTS derivative, 5-Fluoro-FTS (F-FTS), has been previously shown by studies from Prof. Kloog's lab to be a highly potent immunomodulatory drug (11). Thus, as stated in the first year report, we requested Ricerca Biosciences, LLC to prepare ~50 grams of 5-Fluoro-FTS (Concord, OH 44077, USA). Due to raw material issues, the vendor produced and shipped, the lot to us (with all the appropriate certificates of analysis) only during Feb 2016 (month 16 of the project).

Starting mo. 17 of the project, we investigated more broadly the relative therapeutic efficacy of F-FTS in AIA, we conducted a set of large experiments comparing "head-to-head" the clinical effects of F-FTS vs. FTS. The combined results from these studies demonstrate that prophylactic dosing with F-FTS (60mg/kg) from day +1 post CFA injection had a significant therapeutic effect on the clinical outcome of AIA without obvious *in vivo* toxicity. The efficacy of F-FTS was indeed superior compared to FTS treatment ($P < 0.05$ by one-way ANOVA and post-hoc Bonferroni's Multiple Comparison Test (Figure 1).

Responsible PI: Yoel Kloog, Tel Aviv University.

- vi. As described in first year report, in the next set of experiments, we tested the efficacy of FTS when the treatment was given in the "therapeutic scheme", meaning that therapy was initiated at day +9 (onset of arthritis in the hind paws). We found that therapeutic dosing of FTS did not effectively reduce disease severity as measured by both clinical disease scoring and ankle swelling per caliper measurements. As expected, dexamethasone therapeutic dosing (positive control) was effective in decreasing the severity of AIA compared to CMC vehicle treatment ($p < 0.01$). Additionally, in a single exploratory study we also tested FTS as an add-on therapy to MTX (0.5mg/kg) in the therapeutic dosing model compared to this combined treatment in the prophylactic protocol. We found that while prophylactic FTS+MTX dosing almost completely prevented arthritis development, the therapeutic FTS+MTX dosing schedule was only minimally effective on disease outcome measures ($p = \text{N.S.}$). Of note, in the present reporting period, due to the delay in the production of F-FTS by the supplier, we were unable to investigate the efficacy of F-FTS in the therapeutic-dosing-scheme. Although, our observation, as reported previously, that giving MTX (a cornerstone drug for RA treatment) combined with FTS in the therapeutic-dosing-scheme had only limited ($p = \text{N.S.}$) clinical efficacy, implies that this dosing scheme is imprecise for predicting the "true" therapeutic potential of certain agents in RA.

Responsible PI: Yoel Kloog, Tel Aviv University.

Subtask 2B: To analyze the effects of FTS treatment on the cytokine profile and relevant isolated T cell subsets including, TH17, TH1 and foxp3+ regulatory T cells and related molecular markers (**months 1-15**).

- vii. Next, we analyzed the effects of the various treatment protocols on the immune response to CFA, particularly on the CD4+ T cell response. The use of this adjuvant model also offers an opportunity to study the pathological changes in a variety of tissues other than the joints, particularly useful is the splenomegaly that occurs as a marker of the systemic inflammation induced by CFA (9, 10). Thus, at study termination both peripheral blood samples were collected and spleens were harvested. Our first year results, as detailed in the previous report, showed that adjuvant injection resulted in a statistically significant increase in granulocyte percentage, both in peripheral blood and spleen regardless of FTS and/or MTX treatment. In addition, we observed a trend towards an increased CD4 to CD8 T

cell ratio in the spleens of FTS treated rats compared to control rats. This effect was likely due to a statistically significant increase in the percentage of CD4+ Foxp3+ regulatory T cells (Treg) in the spleens of FTS treated rats (see relevant figure in first year report).

Responsible PI: *Itamar Goldstein, Tel Aviv University.*

- viii.** As both TH1 and more recently TH17 cells have been postulated to be instrumental in the pathogenesis of T cell dependent autoimmune responses, both in animal models and humans, we analyzed the effects of FTS and other treatments on the induction of these T cell subsets in arthritic rats. As detailed in the first year report, our data show that FTS therapy was associated with significantly lower numbers of CD3+ CD4+ TH1 and TH17 cells in the spleens of treated rats compared to CMC vehicle treated mice. This effect was even more significant when we administered FTS, as an add-on therapy to MTX. These immunological data positively correlated with the observed clinical outcomes of the various treatment protocols, highlighting the finding that the combined FTS+MTX treatment was associated with a stronger suppression of ankle joint swelling and the pathogenic TH17 response.

Responsible PI: *Itamar Goldstein, Tel Aviv University.*

- ix.** In the first year report, we also investigated the effects of FTS on the pro-inflammatory TH17 response to CFA, measuring the levels of the cytokines IL-17 and IL-6 in serum samples collected at various time points. In accordance with the intracellular cytokine detection data, we found that on day +14 both FTS and MTX alone typically reduced serum IL-17 levels by ~50%, whereas the combined treatment of FTS+MTX reduced IL-17 levels even more significantly by >90%, as compared to CMC control treated arthritic rats. Regarding serum IL-6 levels, in repeated experiments using readymade validated ELISA kits (Rat IL-6 Platinum ELISA from eBioscience, Inc., USA), we could only detect low levels of this cytokine approaching the limit of detection of rat IL-6 (20-30 pg/mL) in all samples of sera tested regardless of the animal treatment protocol and time point (data not shown). During this reporting period we also analyzed serum IL-6 levels using another approach: Luminex xMAP bead-based immunoassay technology (see item **xiv**)

Responsible PI: *Yoel Kloog, Tel Aviv University.*

- x.** IL-6 stimulates the acute phase production of C-reactive protein (CRP) in the liver; hence we measured by ELISA serum levels of CRP as a 'surrogate' marker for CFA-induced systemic inflammation. As detailed in the previous report, we found that CFA injection indeed induced a substantial increase in CRP levels in CMC vehicle treated arthritic rats. In contrast, the combined FTS+MTX completely abolished this increase at the two-time points (day +10 and +14 of the study). While both FTS and MTX reduced CRP levels at day +10, they were less effective, as a single agent, in suppressing the induction of CRP on day +14. Moreover, our CRP data show that it directly correlated with the extent of target

tissue inflammation (ankle diameter) that was strongly inhibited by the combined treatment. **Responsible PI: Yoel Kloog, Tel Aviv University.**

- xi.** To further address the effect of FTS treatment on serum levels of an array of rat inflammation-related cytokines we used the Quantibody array platform (Quantibody Rat Inflammation Array 1, RayBiotech, Inc., USA), a multiplexed sandwich ELISA-based quantitative array platform that enables accurate determination of the concentration of the following cytokines in serum: IFN- γ , TNF- α , IL-4, IL-6, IL-10, IL-1- α , IL-1- β , IL-2, IL-13, and MCP-1. Based on previous studies we analyzed the serum samples from arthritic rats at the onset of disease (day +10) for the levels of these cytokines. However, by this Quantibody Rat Inflammation Array 1, we could only detect low levels for the majority of the analytes, regardless of the AIA treatment protocol (we analyzed selected samples from two different experiments). This precluded us from drawing concrete conclusions on the effects of FTS on these cytokines at this stage. As already discussed in the first year report, the difficulty to reliably detect treatment dependent changes in serum cytokines other than IL-17 by two different methodologies, classical ELISA and multiplexed sandwich ELISA, required us to use a newer Luminex xMAP bead-based immunoassay technology that theoretically should offer higher sensitivity and specificity compared to traditional ELISA based approaches. Unlike traditional ELISA, the Luminex xMAP capture antibodies are covalently attached to the bead surface, allowing for a greater surface area coupled with a free 3-D solution/liquid environment to react with the analytes. Thus we next analyzed the effects of FTS treatment on the serum cytokine using the ProcartaPlex (14plex cytokine array) Rat Th Complete Panel (from eBioscience, Inc., USA). Unfortunately, by this method, we could also only detect threshold levels for all the analytes; this regardless of the AIA treatment protocol and in a large number of selected samples obtained from various independent experiments. This obviously prevents us from drawing relevant conclusions on the effects of FTS on the serum cytokines profile.

Responsible PIs: Itamar Goldstein, Tel Aviv University.

- xii.** Inbred Lewis rats are susceptible to the induction of AIA following immunization with heat-killed *Mycobacterium tuberculosis* (Mtb). The mycobacterial heat-shock protein 65 (Bhsp65) has been implicated in the immune-pathogenesis of AIA (12). To further address the problem of reliably detecting the cytokine profile in the serum of arthritic rats, as detailed above, we decided to use *in vitro* antigenic re-stimulation with Bhsp65 as an additional approach to study the cytokine response. Thus, following immunization with CFA/Mtb, the animals were treated starting day +1 with FTS, F-FTS or CMC vehicle. The rats were sacrificed on day +12, at the onset of clinical arthritis and their spleen and draining superficial inguinal and para-aortic lymph nodes (LN) were harvested for further analysis. Thereafter, a single-cell suspension of a mixture of spleen and LN cells was obtained, and the cells were cultured at 37°C for 3 days in a six-well plate (5×10^6 cells/well) with or without Recombinant MTb Bhsp65 protein (5 μ g/ml), as previously described (13). The supernatants were collected and

analyzed for IL-17 secretion by ready to use high sensitivity ELISA kits (eBioscience, Inc, CA 92121, USA). As shown in Figure 2B, as expected we detected strong induction of IL-17 secretion following *in vitro* antigenic re-stimulation with MTb-Hsp65, chiefly in the T cell cultures of untreated mice. Importantly, *in vivo* prophylactic FTS and F-FTS treatment correlated with significantly reduced induction of with MTb-Hsp65-specific IL-17 producing T cells compared to CMC treated control rats (67.1 ± 1.7 , 11.0 ± 0.7 , and 102.7 ± 1.8 ; $p < 0.001$ for both FTS vs. CMC and F-FTS vs. CMC, by one way ANOVA with post-hoc Bonferroni's Multiple Comparison Test). Of note, F-FTS treatment was moreover more effective in the suppression of the two disease outcome measures (Figure 1) as well as the pathogenic antigen-specific TH17 response (Figure 2). Unfortunately, by parallel analysis of samples from these supernatants with the ProcartaPlex (14plex cytokine array) Rat Th Complete Panel (eBioscience, Inc., USA), we could not detect significant induction of additional relevant cytokines (data not shown).

These data new data further demonstrate that our Ras-inhibitors, particularly F-FTS, prevent effective induction of the pathogenic autoimmune MTb-Hsp65-specific TH17 response, and as expected this immunomodulation is linked with reduced subsequent development of target tissue inflammation.

Responsible PI: Yoel Kloog, Tel Aviv University.

- xiii.** In accordance with revised SOW, to gain a more comprehensive insight into the "molecular mechanism" that mediate the therapeutic action of small molecule Ras-inhibitor in AIA, we analyzed the changes in gene expression (mRNAs) induced by FTS and F-FTS treatment in pure CD4⁺ T cells by gene arrays. As detailed above (item **xiv**), we used *in vitro* antigenic re-stimulation with Bhsp65 to study the effect of the *in vivo* FTS and F-FTS treatment on the CD4⁺ T cell response. Thus, following immunization with CFA/Mtb, the animals were treated starting day +1 with FTS, F-FTS or CMC vehicle. At the onset of clinical arthritis (~ day 12 of the study) spleen and draining superficial inguinal and para-aortic LN were harvested for further analysis. Next, a single-cell suspension of a mixture of spleen and LN cells was prepared, and the cells were either stimulated or not with recombinant Bhsp65 (5 µg/ml) and immediately cultured at 37°C for 3 days in a six-well plate (5×10^6 cells/well). At the end of culture, we magnetically labeled the cells with rat CD4 MicroBeads and then isolated CD4⁺ T cells using the MACS® separation technology (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). High quality total RNA was extracted from the purified CD4⁺ T cells, using Direct-zol™ RNA Kits (Zymo Research Inc., USA). The RNA samples were processed at our Genomics Core Facility, as the input for the amplification and generation of biotin-labeled fragment cRNA for transcriptome analysis using Affymetrix Rat GeneChip® Gene 2.0 ST Arrays, according to the manufacture's guidelines.

We first used hierarchical clustering analysis to group experiments into clusters with similar profiles. As depicted in the dendrogram illustrating the clustering of the samples (comparing columns in the matrix) over heat map of the gene

expression data, we show that the various treatment arms, as expected, clustered together as follows: Tech. replicates < Biol. Replicates < Different treatment groups (Figure 3A). Specifically, the highest distance among samples (quantification of dissimilarity) was connected with *in vivo* FTS treatment and subsequent Bhsp65 antigen *in vitro* re-stimulation (AG) versus control medium (Med). Next, by applying the principal component analysis (PCA) to analyze our gene expression data from the 12 different samples, we found that the experimental conditions that best explain the gene expression patterns were the *in vivo* and *in vitro* treatment protocols of the samples. The dissimilarity among samples was dependent on whether the CD4⁺ T cells were re-stimulated *in vitro* with antigen (AG) as well as whether the rats were treated with FTS/F-FTS or not (Figure 3B). When both experiments and genes are analyzed together, there is a combination of these affects, the utility of which remains to be explored. This report focuses on consideration of the experiments as variables. We first create a covariance matrix to measure how each experiment contributes information to the data set.

The advanced bioinformatics analysis of the various CD4⁺ T cell samples showed that the antigenic re-stimulation with Bhsp65, as expected, induced a robust mixed TH1 and TH17 response in control CMC treated mice. As shown in Figure 3C (heat map analysis), Bhsp65 stimulation induced both IL-22 and IL-17A/F (TH17 response) as well as IFN- γ (i.e., TH1 response) gene transcription. This upregulation was significantly reduced ($p < 0.05$, unpaired t-test) by FTS and F-FTS treatment compared to the CMC control arm. Importantly, these data strongly confirmed the IL-17A-ELISA data, as detailed above, proving that Ras-inhibitors are potent down modulators of the pathogenic TH17 responses in the AIA model of RA.

Next, to determine, in unbiased manner, the biological processes and molecular functions that mediate the therapeutic effect of FTS and F-FTS, we performed additional in-depth bioinformatics analysis. Thus, we computed the overlaps between our gene list and all gene sets in the major collections of the Molecular Signatures Database (MSigDB; developed at the Broad Institute of MIT and Harvard), by means of the Gene Set Enrichment Analysis (GSEA) software and, available online at: <http://software.broadinstitute.org/gsea/msigdb/annotate.jsp>

By this analysis, we determined that the list of genes up regulated significantly (>2-fold change, FDR $q < 0.05$) following Bhsp65 re-stimulation of CD4⁺ T cells isolated from CMC treated control rats exhibited significant overlap with a number of relevant immune and cell proliferation related processes, as follows:

- (i) GO_CYTOKINE_ACTIVITY (FDR=2.39E⁻¹⁸)
- (ii) GO_IMMUNE_SYSTEM_PROCESS (FDR=2.11E⁻¹⁶)
- (iii) HALLMARK_G2M_CHECKPOINT (FDR=3.45E⁻¹³)
- (iv) HALLMARK_INFLAMMATORY_RESPONSE (FDR=1.12E⁻¹¹)
- (v) GO_RESPONSE_TO_TUMOR_NECROSIS_FACTOR (FDR=1.65E⁻⁸)
- (vi) GO_RESPONSE_TO_IFN_GAMMA (FDR=3.22E⁻⁷)
- (vii) GO_POSITIVE_REGULATION_OF_CELL_PROLIFERATION (FDR=3.6E⁻⁷)

- (viii) HALLMARK_IL6_JAK_STAT3_SIGNALING (FDR=5.86E⁻⁴)
- (ix) HALLMARK_K-RAS_SIGNALING_UP (FDR= 4.47E⁻⁰³)

Next, we plotted a heat map depicting the expression levels of 50 genes within this latter gene list across the various cell samples. As seen in this gene expression matrix (Figure 3C), depicting the fold change in the expression of these genes – pertinent to the pathogenic antigen-specific T cell response in AIA– we observed a universal down modulation of their induction, which was particularly notable in the samples from the FTS treatment arm. In conclusion, these data indeed allowed us to gain further insight into the biology behind the effect of FTS and F-FTS; namely down modulation of the *in vivo* induction of a pathogenic TH17 (IL-22 and IL-17 driven) immune response to CFA/Bhsp65.

Responsible PIs: Itamar Goldstein & Yoel Kloog, Tel Aviv University.

In **Major Task 2** of the revised SOW we proposed to validate in collagen induced arthritis mouse model the prophylactic/therapeutic effects of FTS and FTS derivatives.

During month 15 of the project, the USAMRMC Animal Care and Use Review Office (ACURO) finally approved the protocol PR130028.04 entitled, "Studying the Immunomodulatory Effects of Ras-Inhibitors in the Collagen/Adjuvant Induced Arthritis Mice Model," as of 09-DEC-2015 for the use of mice. The Tel Aviv University IACUC had already approved this protocol on 05-NOV-2014.

Subtask 2A: Analyze the prophylactic/therapeutic effects of FTS and F-FTS on the clinical scoring, Histopathology of ankle joints (H&E staining and Immunohistochemistry for relevant biomarkers), and serum IL-17A/IL-6 levels at polyarthritis onset (**months 16-24**).

CIA is the most widely studied animal model of RA, as it shares several pathological features with RA, and Collagen type-II is (CII) is a major antigen in human cartilage, the target tissue of RA. This model has been used widely to identify potential pathogenic mechanisms of autoimmunity relevant to RA, including the role of specific T cell subsets in disease pathogenesis and progression, as well as to design and test new drugs and therapeutic modalities. For example, the CIA model has been instrumental in the testing and development of the new biologically based therapeutics, such as those that target the pathogenic cytokines TNF, IL-6 and IL-17 produced by macrophages and T cells that are the dominant immune cell mediators of RA pathogenesis. CIA can be established in the genetically susceptible, DBA/1 (H-2q) mouse strain, by immunization with CII emulsified in CFA (on study day 0 and 21). The ensuing pathogenesis shares several clinical and immunological features with RA, including synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, and like RA, the disease is dependent on MHC class II genes and T cells. In our hands, in agreement with previous studies, clinically evident arthritis of the ankle joint usually developed ~10 days after the second booster immunization with CII (namely ~30-32 days after the primary immunization), which progressed, in control animals, into severe polyarthritis within a few days.

We employed two dosing models:

- Prophylactic – Begin dosing 3 days before booster immunization until study end.
- Therapeutic – Begin dosing 1 day after arthritis onset (~ day 30) until study end.

Clinical Assessment:

Disease progression was assessed by a clinical scoring index (0-16 scale) starting at arthritis onset and subsequently every other day until the study termination.

- To determine whether prophylactic dosing with FTS attenuates the clinical signs of CIA, we started daily treatment of mice from day +18 post CFA immunization, 3 days before the booster immunization. We treated the mice in the experimental arms with oral FTS (100 mg/kg) by gavage. Control rats received oral solution of 0.5% CMC vehicle. As a "positive control", we also treated a group of mice with weekly i.p injection of MTX (1.0 mg/kg) a proven RA therapy. CIA severity was scored using a clinical index of 0 to 16 (0-4 scale for each paw). As shown in Figure 4, we found that the arthritis scores were significantly reduced in the FTS treatment group compared to CMC vehicle treated control mice ($p < 0.0001$, by ANOVA with post-hoc Bonferroni's Multiple Comparison Test). Importantly, we also found that although MTX was highly effective therapy ($p < 0.01$, MTX vs. CMC), the magnitude of effect of FTS treatment on the clinical disease outcome measure was higher ($p < 0.05$, MTX vs. CMC, by unpaired t-test).

Responsible PIs: Itamar Goldstein & Yoel Kloog, Tel Aviv University.

- For Histopathological Assessment at study termination, the tibio-tarsal joint was transected at the level of the medial and lateral malleolus. Next, we collected the ankle joints into 4% paraformaldehyde for at least 24 hours and then placed them in a decalcifier solution. When decalcification was completed, we transected the ankle joints in the longitudinal plane and the joints were paraffin embedded, sectioned and stained with hematoxylin & eosin. We are presently in the process of assessing and scoring these joint sections, on a scale of 0-5 for inflammation and bone resorption, by an experienced pathologist blinded to the animal treatment protocol, as previously described (10). We are also in the process of doing immunohistochemistry assessment of deparaffinized and rehydrated arthritic mice ankle joint sections for the infiltrations of T lymphocyte into the synovial tissue, to be detected using anti-CD3 antibodies. We expect to finalize the histopathological assessments of the relevant CIA model joint tissue sections within the first 3-6 months of third year.

Responsible PI: Itamar Goldstein, Tel Aviv University.

- High IgG autoantibody levels against to CII, as well as the antibody subtype are important for inducing arthritis (14). Therefore, the titers of anti-CII antibody are a very useful parameter/marker to probe for effective immunization and the induction of T cell-dependent CII-specific autoreactive pathogenic B cells. We used a readymade "gold standard" anti-CII IgG ELISA kit (Chondrex, Inc. Redmond, WA 98052) to analyze the serum of the various mice groups for titers of these autoantibody. Our results (Figure 5A) clearly demonstrate that both FTS

and MTX significantly inhibited the antibody response to the CII immunization ($p < 0.01$, for both FTS vs. CMC and MTX vs. CMC, by unpaired t-test). Importantly, these data imply that FTS treatment is non-inferior to the cornerstone DMARD widely used and generally effective in a large percentage of RA patients, MTX. **Responsible PI: Itamar Goldstein, Tel Aviv University.**

- iv. The pro-inflammatory cytokine IL-6, actually, regulates the acute phase production of CRP in the liver as well supports the induction of an autoantigen-specific TH17 response. Moreover, both IL-6 and IL-17 have been shown to be very sensitive and specific markers of systemic inflammation and arthritis mice (14, 15)}. By readymade ELISA kits (all from eBioscience Inc.), we observed at ~3 days post arthritis onset a significant upregulation of IL-6 and IL-17 serum levels in the CMC control treatment arm (Figure 5B). Importantly, we discovered that the induction of cornerstone cytokine IL-6 was significantly inhibited in the FTS, F-FTS, and MTX treatment arms, and more importantly the induction of IL-17 was completely blocked. This observation implies that FTS/F-FTS are strong inhibitor of the systemic inflammatory response post-adjuvant/CII injection in mice consistent with the relevant cytokine data from the AIA rat model of RA. **Responsible PI: Yoel Kloog, Tel Aviv University.**

Subtask 2B: Analyze the effects of the Ras inhibitors on relevant immune cells and T cell subsets including FOXP3⁺ Treg cells, relevant molecular markers such p-AKT and p-ERK, and the cytokines response by multiplex analysis (mo. 16-36).

- v. To analyze the effects of the various treatment protocols on the immune response to CFA/CII, particularly on the T cell response, at study termination we harvested spleens and prepared single cell suspensions'. The cells were immunostained for CD3+CD4+ and CD3+CD8+ T cells and granulocytes (Gr1+ CD11b+). We found that adjuvant/CII immunization resulted in a robust reduction of CD3+ T cells numbers in the spleen compared to naïve mice, regardless of the specific treatment. We also observed a statistically non-significant (ns) trend of reduced CD4+ to CD8+ T cell ratio in the FTS treatment arm (Figure 6A/B). Importantly, we found (Figure 6B) a significant increase in granulocyte percentage in the spleens of FTS and MTX active treatment arms vs. CMC control ($P < 0.05$ for both comparisons by Student's *t*-test). **Responsible PI: Itamar Goldstein, Tel Aviv University.**
- vi. Foxp3+ Treg cells suppress autoimmune responses, both in animal models and humans. Thus, we analyzed the effects of FTS and other treatments on the induction of this immunoregulatory T cell subset in CIA. Our data show that FTS therapy was associated with significantly increase in Foxp3+ Treg cells in the spleens of treated rats compared to CMC vehicle treated mice (Figure 7). **Responsible PI: Itamar Goldstein, Tel Aviv University.**
- vii. Activation of the RAS signaling is marked by elevated levels of p-ERK as well as p-AKT, dependent on the cellular context. For example, ERK activation by RAS

can regulate T-cell sensitivity to antigenic stimulation. Increased pERK activity sustains several positive feedback loops important for T cells activation, survival and proliferation. Inhibition of this molecular pathway is associated with T cell anergy and reduced IL-2 production (2). Importantly, T cells isolated from synovia of RA patients expressed higher levels of K-Ras, B-Raf and their downstream effector p-ERK1/2 (3, 4). Therefore, to verify that FTS therapy was indeed associated with effective blockade of the RAS signaling cascade in lymphocytes, we analyzed p-ERK and p-AKT levels in CD4⁺ T cells from spleen of CII immunized mice at necropsy, as a molecular marker for effective targeting of Ras signaling. By the by Phospho Specific Flow Cytometry, we show that both p-ERK and p-AKT levels were significantly reduced (Figure 8A), *in vivo*, in freshly isolated splenic CD4⁺ T cells. Our western blotting analysis (WB) also show significantly reduced Ras-GTPase levels in the extracts of splenic lymphocytes from CII-immunized mice treated with FTS or FTS+MTX compared to MTX only or CMC treated mice (P<0.01 by ANOVA and post-hoc Bonferroni's Multiple Comparison Test). These data validate that the good clinical efficacy of FTS alone or as an add-on to MTX correlated positively with reduced Ras signaling (Figure 8A) and levels (Figure 8B/C) in splenic lymphocytes.

Responsible PI: Yoel Kloog, Tel Aviv University.

- viii. T cells play a crucial role in CIA, chiefly by the induction of a T cell dependent autoreactive B cell response to type II collagen (CII) and the consequent production of high affinity anti-CII autoantibodies. To gain a comprehensive insight into the effect of FTS treatment on the CII-specific T cell response and T-helper (TH) polarization, we investigated in-depth the cytokine response, using the Luminex xMAP bead-based multiplex immunoassay technology. Thus, we first prepared a single cell suspension from spleen and lymph node cells of mice immunized with CII and treated with oral FTS or CMC vehicle. The various cell samples were immediately stimulated with the relevant T cell pathogenic antigen, heat denatured bovine CII (from Chondrex Inc.), and the cells were cultured for 72 hours. At the end of culture, the supernatants were collected and we determined the induction of a large number of relevant cytokines, using the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel (17 plex), per manufacturer's recommendations. We found that the re-stimulation of CII-specific effector T cells from immunized untreated arthritic mice (CMC control treatment arm) induced a strong upregulation of the TH17 associated cytokines (IL-17A and IL-22), the TH1 cytokine IFN- γ , and the TH9 cytokine IL-9. FTS treatment was associated with down modulation of this array of arthritis-inducing cytokines in respective cultures, as compared to T cell cultures of control mice. Furthermore, FTS treatment also caused significant inhibition of the CII-stimulation-dependent induction of the pathogenic pro-inflammatory cytokines, IL-6, TNF and GM-CSF (Figure 9).

IL-17 is produced by a distinct subpopulation of CD4⁺ helper T cells that has been designated TH17 (16). Abundant evidence indicates that T cells are required for initiation and/or chronicity both in human rheumatoid arthritis (RA) and in

mouse models of RA. IL-17 has emerged as a critical T cell cytokine in the pathogenesis of human RA and in several mouse models of this disease. IL-17 neutralization with antibodies or genetic ablation of IL-17 or IL-17R is associated with the attenuation of murine arthritis in several experimental murine models of arthritis (17). Th17 cells also secrete IL-22, a cytokine generally considered pro-inflammatory because of its co-expression with IL-17 by TH17 cells. In the context of RA, mice that are deficient in IL-22 are less susceptible to CIA and/or develop a less severe disease. Moreover, levels of IL-22 are elevated in the periphery and synovia of RA patients (18, 19), and IL-22 can induce proliferation of synovial fibroblasts and promote the generation of osteoclasts. More recently, it was reported that IL-22 plays a pathogenic role during the initiation phase in CIA (18, 19).

Thus, our present cytokine data from the CIA mouse model and our gene arrays data from the AIA rat model are consistent with a pathogenic role for the TH17 related cytokines IL-17A/F and IL-22 in the pathogenesis of murine arthritis. Specifically, these data strongly imply that the antigen-specific T cell response in these two murine models of arthritis is associated with a TH17 response, and more importantly that the therapeutic effect of Ras-inhibitors is very likely due to their ability to attenuate, following immunization, the polarization of the T cell response into a predominantly TH17/TH22 response.

Responsible PIs: Itamar Goldstein & Yoel Kloog, Tel Aviv University.

Milestone #1: Co-authored manuscript on the therapeutic value of Ras inhibitors in the AIA and CIA animal models

As detailed in this report and previous report, we have generated multiple clinical, immunological and molecular data clearly showing that the small molecule Ras-inhibitors, FTS and F-FTS, are potent disease modifying drugs in two major animal models of RA. We are presently in the process of preparing the manuscript that summarizes our novel findings. We recently submitted an abstract entitled "RAS SIGNALING INHIBITION ATTENUATES ARTHRITIS IN ANIMAL MODELS OF RHEUMATOID ARTHRITIS BY DOWN MODULATING THE PATHOGENIC TH17 CELL RESPONSE" for oral presentation in the upcoming European League against Rheumatism (EULAR) 2017 meeting in Madrid.

Responsible PIs: Itamar Goldstein & Yoel Kloog, Tel Aviv University.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Treatment with FTS in the prophylactic protocol significantly reduces (~ 50% inhibition) the clinical outcome of AIA as assessed by clinical scoring and ankle swelling by caliper measurements. This therapeutic efficacy of FTS treatment correlated with significant reduction in joint inflammation, bone resorption in the ankle joints, and synovial infiltration by CD3+ T cells per the histopathological assessment (**first year and second year reporting periods**).

- Treatment with FTS as an add-on to low dose MTX in the prophylactic dosing scheme significantly increased the therapeutic efficacy of FTS in the AIA model, causing a strong suppression of arthritis development (~90% inhibition), as assessed by clinical scoring and ankle swelling by caliper measurements. The high efficacy of this combined therapy also significantly correlated with inhibition of joint inflammation, bone resorption in the ankle joints, and synovial infiltration by CD3+ T cells per the histopathological assessment (**first year and second year reporting periods**).
- FTS prophylactic treatment was associated with a significantly reduced induction of TH17 cells and a reciprocal increase in the percentage of Foxp3+ Treg cells in the spleens of mice in the FTS arm compared to the CMC control arm, as assessed by FACS based analysis. FTS prophylactic therapy was associated with attenuated increase of serum IL-17 and of the IL6-dependent acute phase reactant, CRP, both at the onset of AIA and at the peak of the disease, as assessed by commercial ELISA kits. The superior efficacy of the combined FTS+MTX therapy indeed correlated with an almost complete inhibition (~90% reduction) of the upregulation of IL-17 and CRP compared to ailing arthritic mice in the CMC control arm of the study (**first year and second year reporting periods**).
- Ricerca Biosciences synthesized a large quantity of the FTS derivative, 5-Fluoro-FTS (F-FTS) enabling us to comprehensively assess its therapeutic efficacy in AIA. Comparing "head-to-head" prophylactic dosing with F-FTS (60mg/kg) versus FTS (100 mg/kg) we determined that F-FTS has superior therapeutic clinical compared to FTS treatment, as assessed by the clinical score of arthritis severity. As detailed below this high therapeutic efficacy positively correlated with its "molecular" immunomodulatory effects (**second year reporting period**).
- FTS and F-FTS treatment induced a significant inhibition of the MTb-hsp65 (Bhsp65)-specific TH17 response observed in control arthritic rats, as assessed by *in vitro* analysis of the T cell recall response to BHsp65. Importantly, the superior clinical efficacy F-FTS treatment positively correlated with its superior capacity to down modulate the pathogenic antigen-specific TH17 response (i.e. reduced IL-17 secretion). These data new data further demonstrate that our Ras-inhibitors, particularly F-FTS, prevent effective induction of the pathogenic autoimmune MTb-Hsp65-specific TH17 response, and as expected this immunomodulation is linked with reduced subsequent development of target tissue inflammation (**second year reporting period**).
- We performed in-depth analysis of the gene expression networks that govern the T cell response to Bhsp65 during the induction of AIA, by comparing the CD4+ T cell response to *in vitro* re-challenge with this antigen. The detailed bioinformatics of the Affymetrix rat GeneChip® arrays data allowed us to gain further insight into the immune-biology behind the therapeutic effects of FTS and F-FTS. We found that our Ras-inhibitors are potent down-modulators of the *in vivo* induction of gene sets regulating the pro-inflammatory IL-22 and IL-17 mediated (TH17) immune

response to the arthritogenic microbial antigen Bhsp65 (**second year reporting period**).

- We determined that prophylactic dosing with FTS had a non-inferior therapeutic effect as compared to i.p injection of MTX a recognized therapy for CIA and RA. In addition, we show that prophylactic dosing of FTS as an add-on to MTX showed no improved clinical efficacy as compared to FTS treatment alone. Actually, the magnitude of the therapeutic effect of FTS treatment on the clinical disease outcome measure was the highest compared to the other active treatment arms (**second year reporting period**).
- FTS prophylactic treatment alone or as an add-on to MTX as well as MTX treatment alone were all associated with significant "positive" attenuation of relevant laboratory markers of inflammation and/or arthritis severity. We determined that both the robust upregulation of the serum levels of anti-CII autoantibodies and of the cytokines IL-6 and IL-17A observed in control mice, were significantly inhibited by each of the active drug regimens. Importantly, FTS alone was as effective in suppressing the induction of these markers of disease activity, as compared to MTX alone or combined with FTS, confirming that FTS was non-inferior compared to MTX both in clinical and laboratory outcomes (**second year reporting period**).
- FTS therapy was indeed associated with reduced p-ERK and p-AKT levels in splenic CD4+ T cells and of total Ras levels in spleen samples of CII immunized mice. Thus, we validated that the good clinical efficacy of FTS alone or as an add-on to MTX correlated positively with its predicted molecular effect (**second year reporting period**).
- The in depth multiplex analysis of the T cell cytokine response to CII antigen re-challenge are consistent with a pathogenic role for the TH17 related cytokines (i.e., IL-17A and IL-22). They also validate our working hypothesis that the biology behind the immunomodulatory effect of FTS and F-FTS is primarily attenuation of the induction of a pathogenic TH17 immune response following CFA/CII immunization (**second year reporting period**).

5. CONCLUSION:

During the first two year of the award, we reached a few major conclusive findings with significant medical implications:

- (I) Prophylactic treatment with the small molecule FTS, a first-in-class oral selective inhibitor of RAS signaling, provides a significant immunomodulatory effect in the AIA model by all outcome parameters (Clinical scoring, Ankle joint swelling, histopathological assessment, serum markers of inflammation, tissue levels of RAS-GTP and p-ERK, etc.), and
- (III) The remarkable discovery that prophylactic dosing of FTS as an add-on to MTX provides a very strong protective effect (~90% effect) against the development of AIA by all relevant clinical and laboratory outcomes parameters.

(II) F-FTS showed superior therapeutic clinical efficacy compared to FTS, as assessed by the clinical score of arthritis severity and certain relevant laboratory parameters.

(III) The detailed bioinformatics analysis of the Affymetrix rat GeneChip® arrays data allowed us to gain further insight into the true biology behind the therapeutic effects of FTS and F-FTS, discovering that these compounds are potent down-modulators of the in vivo induction of IL-22 and IL-17 mediated (TH17) immune response to Bhsp65 as well as IL-6/TNF mediated inflammation.

(IV) Prophylactic dosing with FTS had a non-inferior therapeutic effect as compared to i.p injection of MTX a mainstay DMARD in the mouse CIA model.

(V) FTS prophylactic treatment alone or combined with MTX was coupled with significant "positive" attenuation of multiple relevant laboratory markers of inflammation and surrogate molecular markers of Ras signaling. Importantly, the in depth analysis of these data validated our working hypothesis that the biology behind the immunomodulatory effect of FTS and F-FTS is inhibition of a pathogenic TH17 immune response following CFA/CII immunization.

Our future aims are to accomplish the goals and tasks, as described in the original project narrative and the approved revised SOW. Briefly, our plans for the third year of the award are as follows:

(A) To test in the mouse CIA model the efficacy of the therapeutic dosing scheme of FTS and the efficacy of the prophylactic/therapeutic dosing schemes of F-FTS.

(B) To gain additional comprehensive mechanistic insight into the biological processes and molecular functions that mediate the immunomodulatory effects of FTS and F-FTS. We plan to assess a wide range of mouse T cell functions and biologically relevant molecular pathways, both in vivo during immunization to CII and in vitro in relevant pure T cell subset.

(C) To publish a comprehensive manuscript summarizing our extensive data from the AIA and CIA studies. In this report, we aim to elucidate the role Ras-signaling in pathogenesis of arthritis and provide relevant mechanistic insight on the effects of small molecule Ras inhibitors in the AIA and CIA models of RA. The manuscripts will include also data and conclusion derived from the in vitro work detailed in Task 4 (To analyze in vitro the effects of FTS on signaling events following T cell antigen receptor stimulation).

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the first year:

Manuscript in preparation as detailed in Milestone #1 (p. 17).

b. List presentations made during the first year

An abstract entitled "RAS SIGNALING INHIBITION ATTENUATES ARTHRITIS IN ANIMAL MODELS OF RHEUMATOID ARTHRITIS BY DOWN MODULATING THE

PATHOGENIC TH17 CELL RESPONSE", was submitted for oral presentation in the upcoming European League against Rheumatism (EULAR) 2017 meeting in Madrid.

7. INVENTIONS, PATENTS AND LICENSES:

“Nothing to report.”

8. REPORTABLE OUTCOMES:

We provide herewith a list of reportable outcomes representing a scientific advance that makes a meaningful contribution toward the understanding and treatment of Rheumatoid Arthritis.

- Prophylactic treatment with the small molecule oral inhibitor of RAS signaling, FTS and its derivative FTS, provides a significant therapeutic effect both in the rat AIA model and mouse CIA model. This conclusion is supported by clinical and laboratory disease outcome measures, including: clinical score, ankle swelling, histopathological assessment, serum IL-17 and IL-6/CRP levels, percentage of splenic TH1 and TH17 cells and target tissue levels of RAS-GTP and p-ERK/p-AKT. This reportable outcome supports our understanding of T cell dependent autoimmune processes (including RA) by showing a vital role for the Ras-ERK cascade in the development of experimental arthritis. It also supports our view that FTS and/or its derivatives should be further developed and tested as a novel class of targeted synthetic DMARDs.
- Prophylactic treatment with FTS as an add-on to MTX showed a very strong protective effect against the development of AIA by all the outcome measures (as detailed above) that was moreover significantly better than FTS or MTX therapy alone. Importantly, this new combination therapy practically suppressed all clinical sign of disease and the upregulation of serum markers of inflammation (CRP and IL-17 levels). In the CIA model, the therapeutic efficacy of FTS was high such as that combining it with MTX did not provided additional protective effects, yet the combined therapy was not associated with added toxicity. These data are particularly relevant to modern RA therapy, as methotrexate is presently the "cornerstone of therapy" and is likely used in most RA patients treated at Veterans Health Administration's primary care and specialty clinics. For example, most of the new generation RA drugs (biologics and targeted synthetic DMARDs) are routinely prescribed as an add-on therapy to MTX. Thus, this reportable outcome strongly implies that FTS similar to other anti-RA drugs can be used in combination with MTX with improved efficacy and no added toxicity.

These reportable outcomes are promising with respect to their potential to influence the future drug treatment of RA patients in the Veterans Health Administration system.

9. OTHER ACHIEVEMENTS: “Nothing to report.”

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11. APPENDICES:

Attached Figures (9 figures with legends)

Figure 1

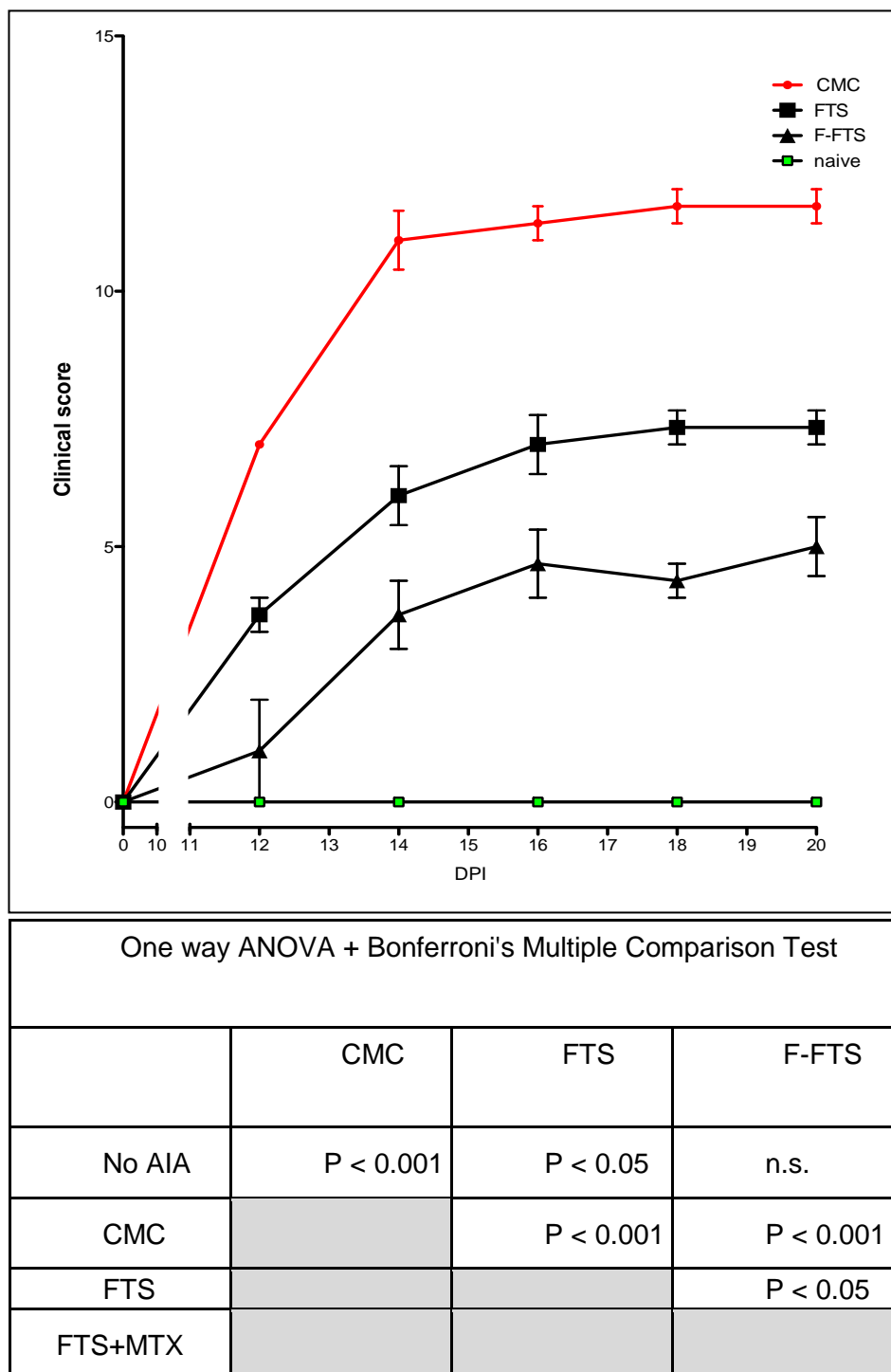


Figure 1: 5-Fluoro-FTS(F-FTS) show higher therapeutic efficacy on disease progression in AIA compared to FTS. Clinical score of AIA severity was graded starting from day +10 (usual time point of arthritis onset in ankle joints). Rats (n=10 per group) were treated daily starting day +1 post CFA injection (prophylactic dosing) with FTS (100mg/kg), 5-Fluoro-FTS (60mg/kg) or CMC vehicle. Statistical analysis between the groups of the results was performed using *One way ANOVA and post-hoc Bonferroni's Multiple Comparison Test* and the comparison is summarized in the table inset. The results shown represent a study out of >3 performed.

Figure 2

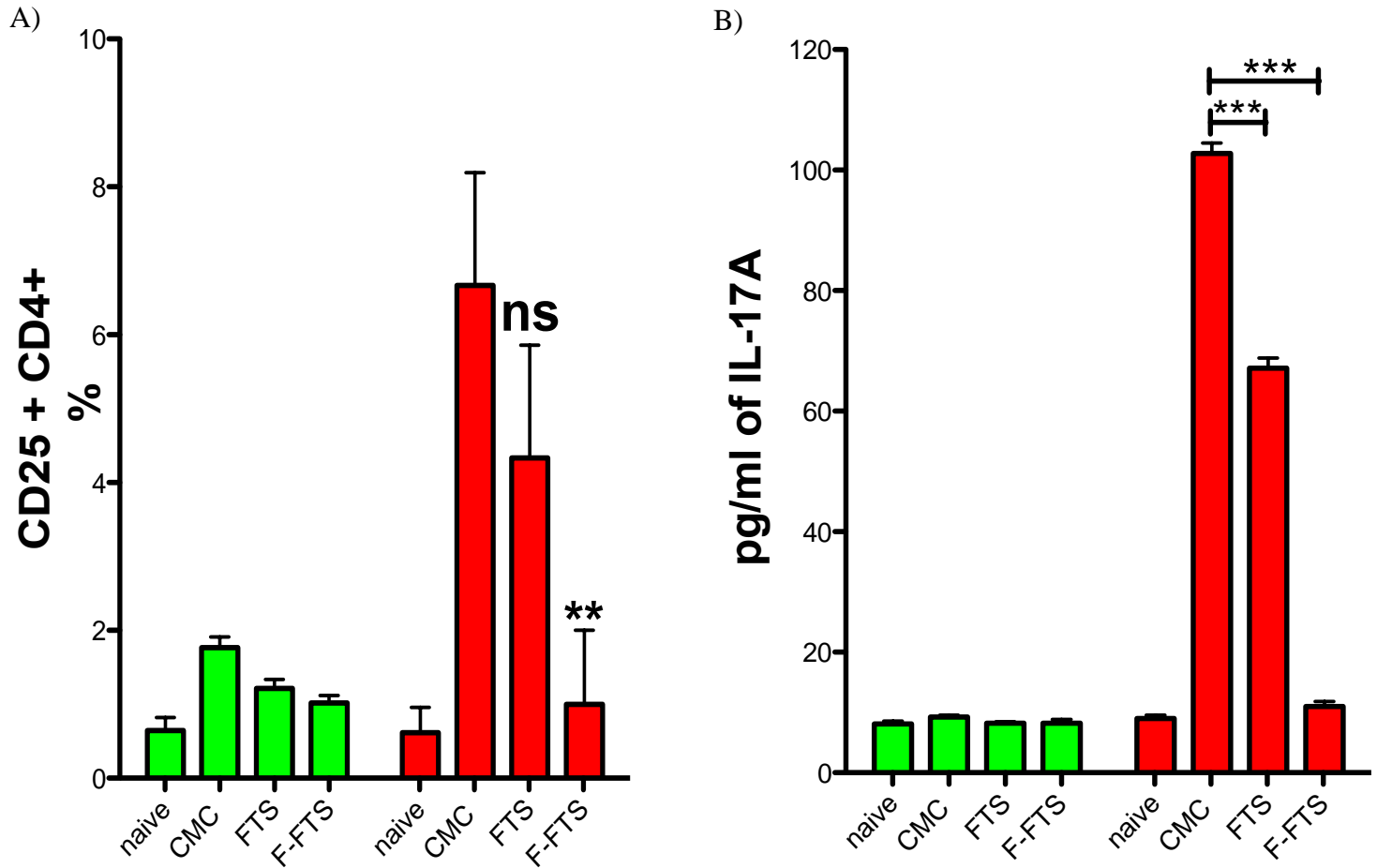


Figure 2: FTS and F-FTS in vivo treatment down modulate the in vitro antigen specific T cell response to BHsp65. Following CFA injection rats were treated with CMC (vehicle), FTS (100mg/kg) or F-FTS (60mg/kg) starting from day +1 post AIA induction. A single suspension cells from LN and Spleen (harvested on day +14 post disease induction) cultured at 37°C for 72 h in RPMI and stimulated with 5µg/ml of Bhsp65 (red bars) or control medium (green bars). (A) At culture end T cells were immunostained with anti rat CD3, CD4 and CD25 mAbs and analyzed by FACS for the percentage of antigen activated CD25+ CD4+ T cells. (B) Supernatants from the same cultures were tested by ELISA kits for levels of secreted 17A by ELISA (red with antigen or with out antigen green). One representative experiments out of two is shown.

Figure 3

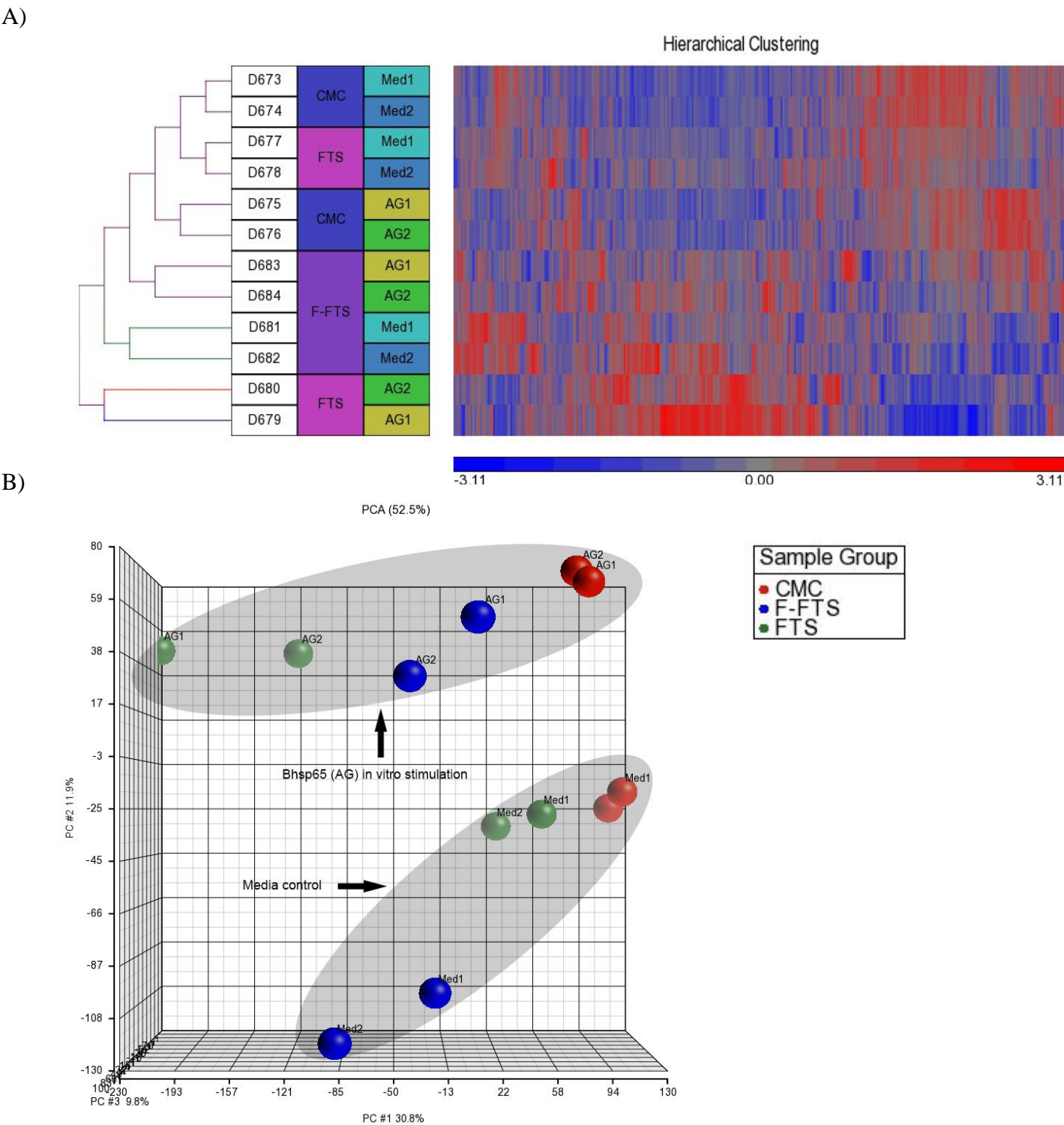


Figure 3

C)

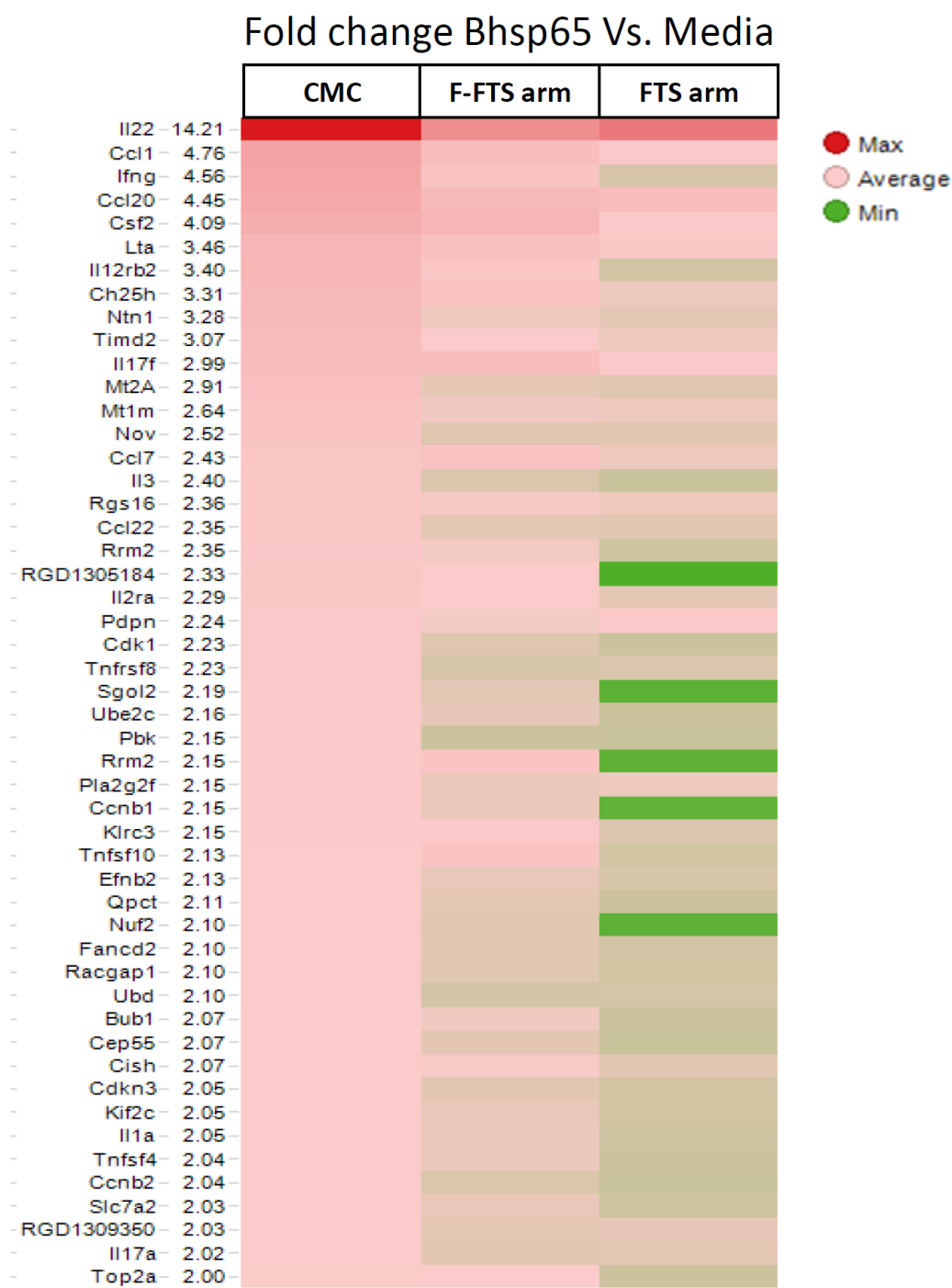
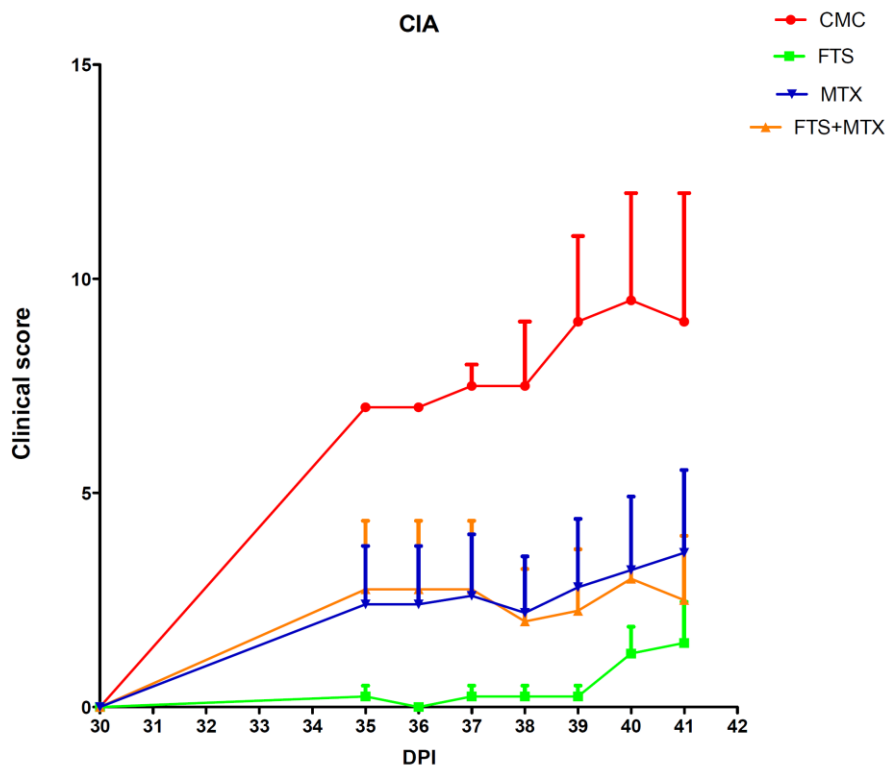


Figure 3: Comparative gene expression profiles of antigen specific T cell response to BHsp65 in vitro after FTS and F-FTS in vivo treatment. A) Hierarchical clustering analysis to group experiments into clusters with similar profiles. **B)** principal component analysis (PCA) to analyze our gene expression data from the 12 different samples. **C)** heat map depicting the expression matrix of the 50 genes significantly up regulated (>2FC) in the FTS treatment group as compared to the other treatment groups

Figure 4



One way ANOVA + Bonferroni's Multiple Comparison Test				
	CMC	FTS	FTS+MTX	MTX
CMC		P < 0.001	P < 0.05	P < 0.05
FTS			ns	ns
FTS+MTX				ns

Figure 4: Prophylactic treatment with FTS has a protective effect on the severity of collagen-induced arthritis (CIA). 8-week-old DBA/1 male mice were given an initial injection of CFA/type II collagen on day 0, and arthritis was induced with a booster immunization with IFA/CII on day 21 of the study. Mice in the experimental arms (n=8 per group) were treated semi-prophylactically, starting from day +18, with either oral FTS (100 mg/kg), weekly i.p injection of MTX (1 mg/kg), FTS combined with MTX, or 0.5% CMC vehicle solution (control group). Clinical score of CIA severity (0-16 scale) was graded starting from day +30 (usual time point of arthritis onset in ankle joints). Statistical analysis of the effect of the various treatments on the clinical scoring was done using the One way ANOVA with post-hoc Bonferroni's Multiple Comparison Test, and the results are summarized in the table inset. This analysis also confirmed that treatment with FTS alone was as protective as MTX=FTS compared to CMC in this RA model. Data are from a representative experiment out of 3 performed.

Figure 5

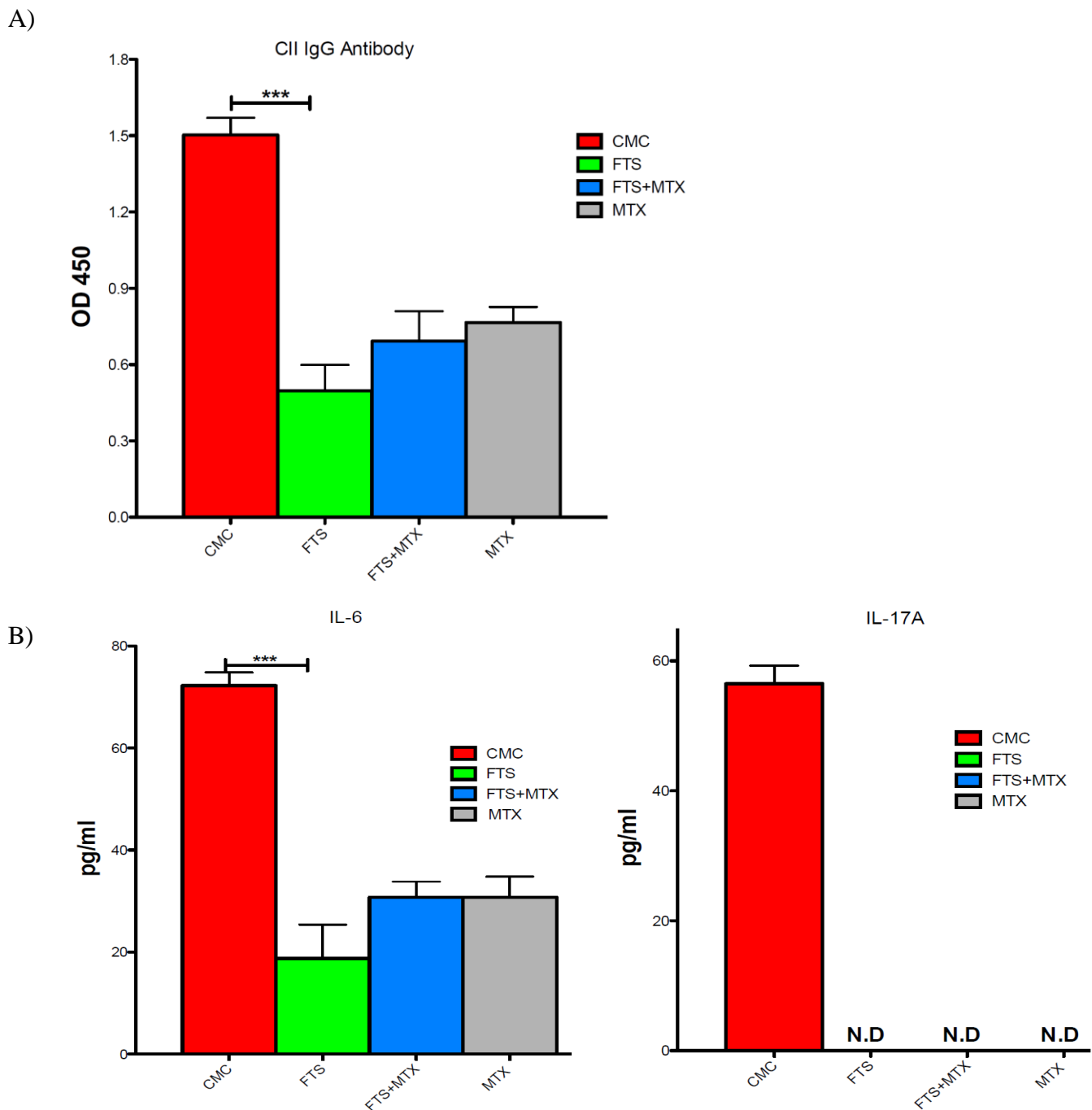


Figure 5: FTS reduces the production of pathogenic anti-collagen antibodies and inflammatory cytokines in CIA. Sera from mice treated with FTS, FTS+MTX MTX, or CMC vehicle were collected at necropsy (day 42 of the study) and analyzed **(A)** by commercial ELISA kits for the level of circulating anti-collagen antibodies. The data are presented as optical density (OD) at 450 nm. Bars represent mean \pm S.E.M of triplicates ($P < 0.001$ for all individual active treatment arms vs. CMC, by t -test). **(B)** Sera from similarly treated mice were obtained at day 35 post immunization and analyzed for IL-17A and IL-6 levels by ready made ELISA kits. Bars represent mean \pm S.E.M of triplicates from a representative experiment out of 3 performed ($P < 0.001$ for all individual active treatment arms vs. CMC, by t -test).

Figure 6

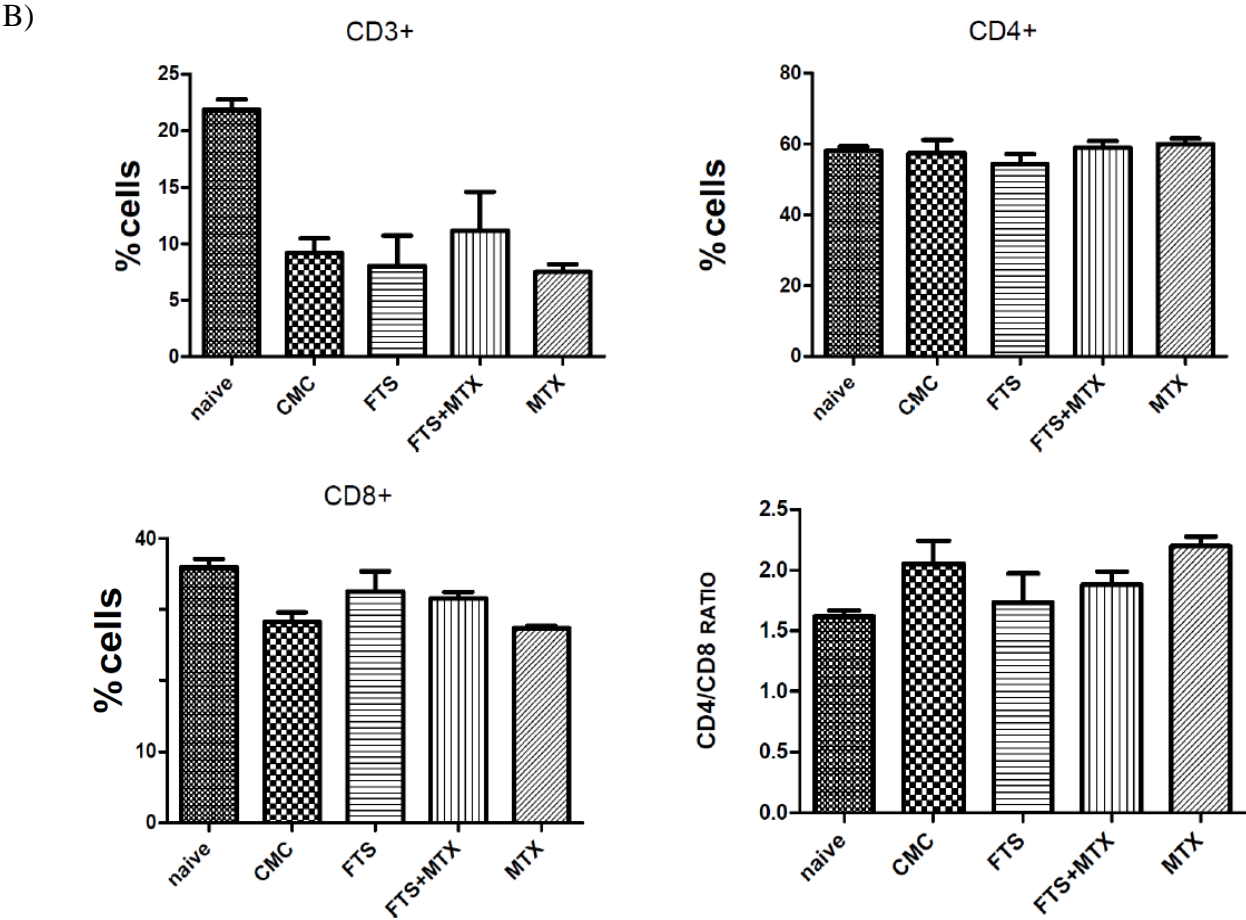
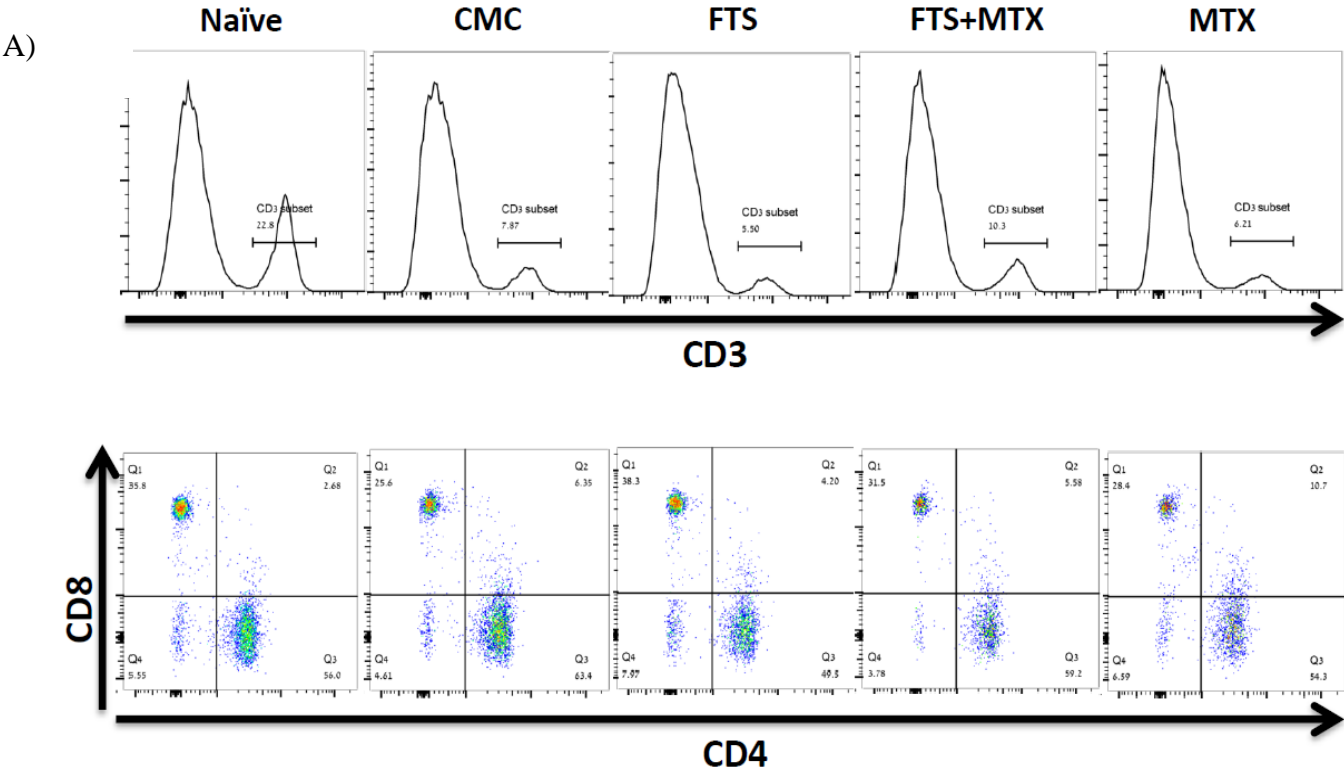


Figure 6

C)

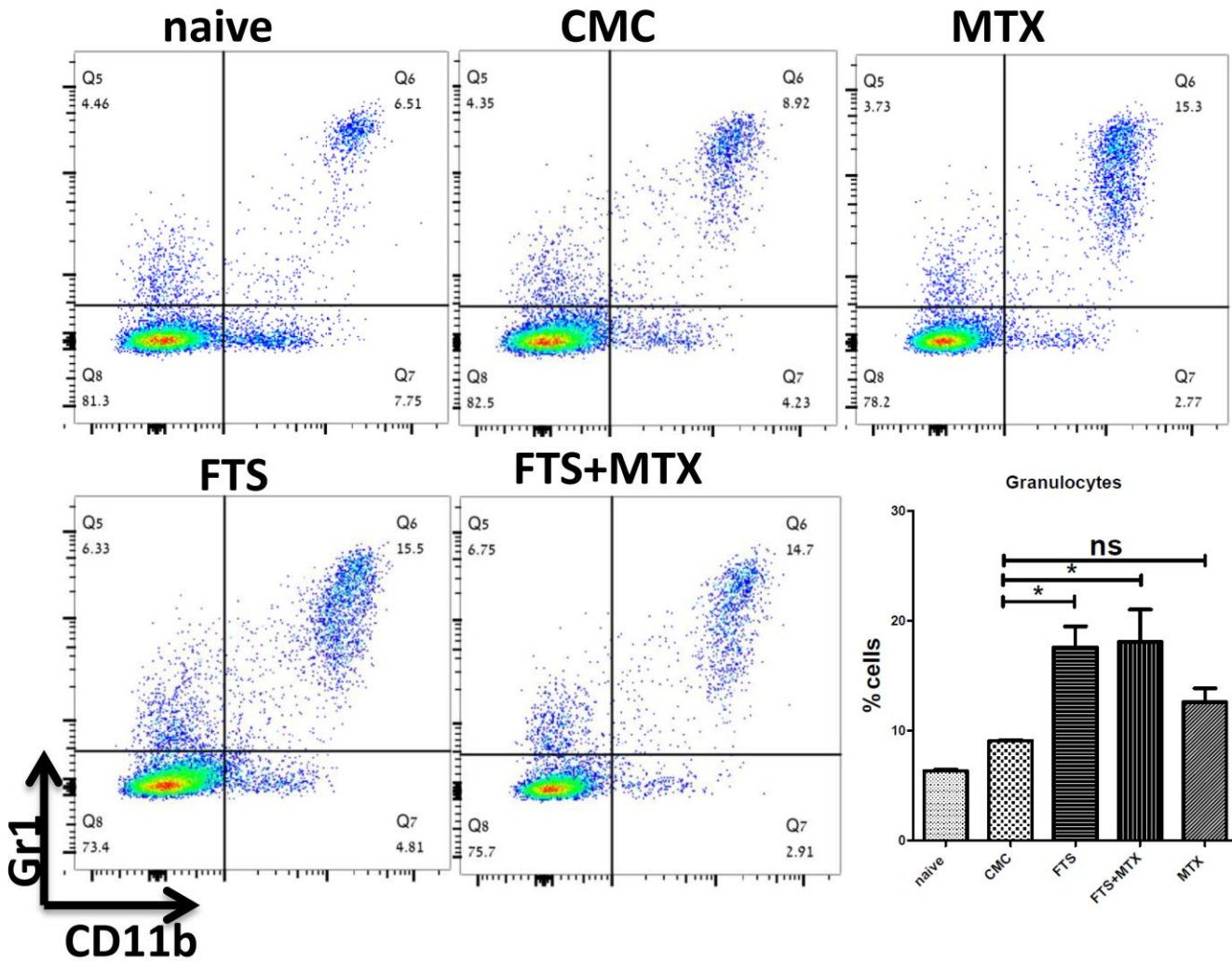


Figure 6. FACS analysis of effects of FTS therapy on T cell populations and granulocytes in the Spleen of CII immunized mice. At study termination (day +42) we immunostaining single cell suspension of harvested spleens with relevant mAbs and subsequently analyzed them by flow cytometry for changes in CD3+, CD4+ and CD8+ T cells (A-B) and (C) Granulocytes. Cells were isolated, as indicated, from mice treated with either FTS, FTS+MTX, MTX and CMC vehicle, as well as naïve untreated mice. The data were acquired on a FACSARIA instrument (~10,000 single cell events) and analyzed using FlowJo software. Bars represent mean \pm S.E.M arm ((n=6 per group). The results are from a typical experiment out of >3 performed. $P < 0.05$ for comparison of granulocyte numbers of FTS and MTX active treatment arms vs. CMC control (by t-test).

Figure 7

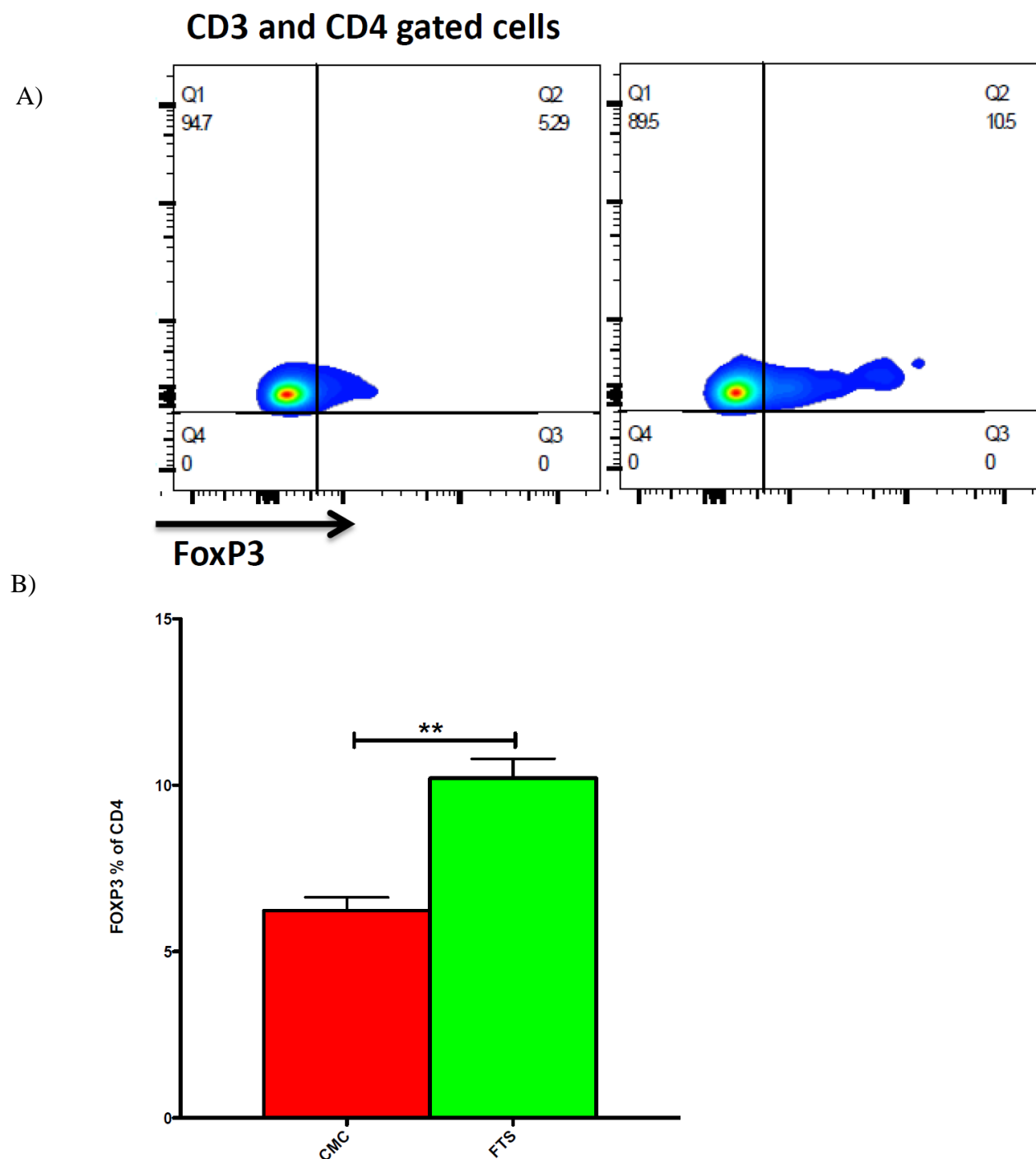
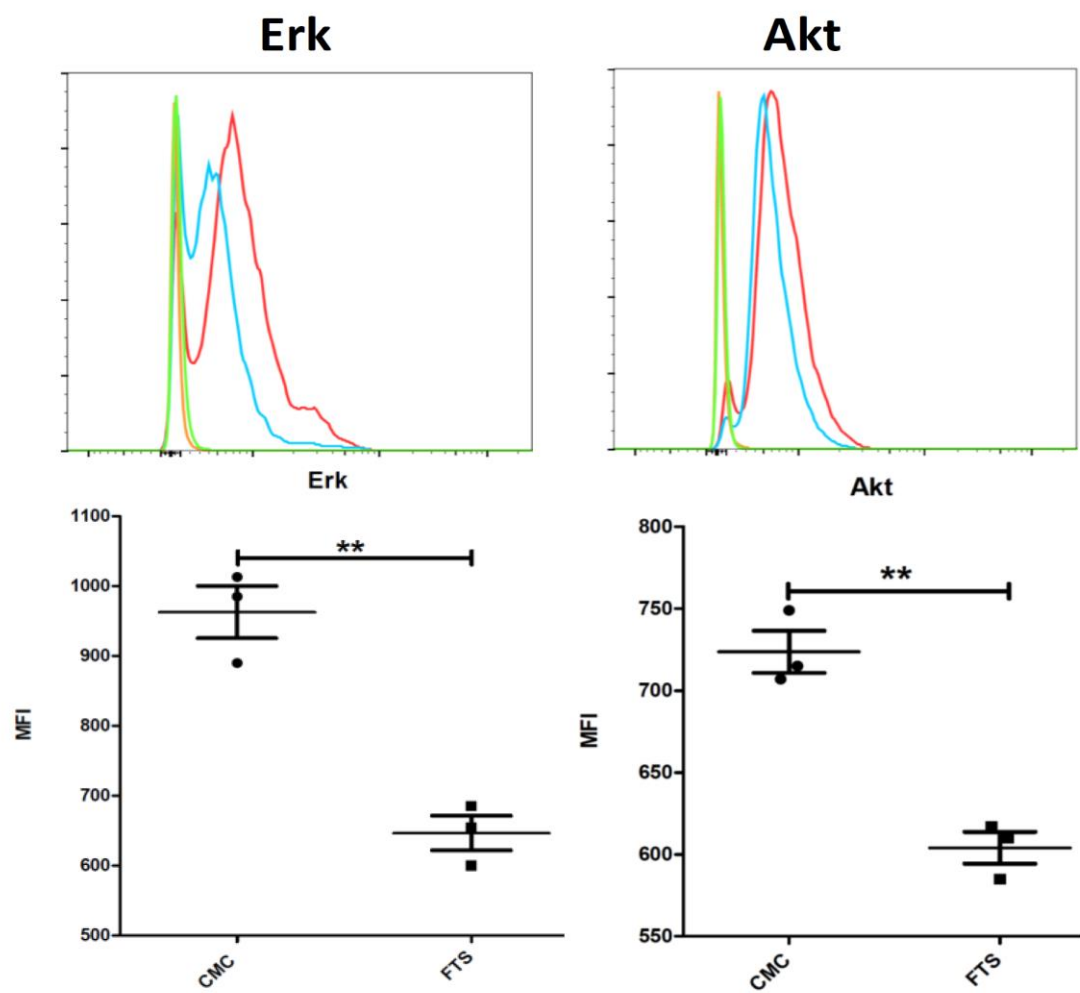


Figure 7. FTS treatment increases regulatory T cell (Tregs) percentage during CIA progression. (A) Representative FACS density plot from an FTS treated (right) vs. CMC control (left) mouse immunostained with fluorochrome-conjugated anti-rat CD3, CD4 and FoxP3 mAbs. The cells were processed from spleens harvested on day 42 of the study. **(B)** Combined data and statistical analysis of Foxp3+ Treg percentages in spleen, and shown are the mean \pm SD of $n=3$ biological replicates ($P<0.01$ for FTS treatment arm vs. CMC control by t -test).

Figure 8

A)



B)

Ras levels by WB

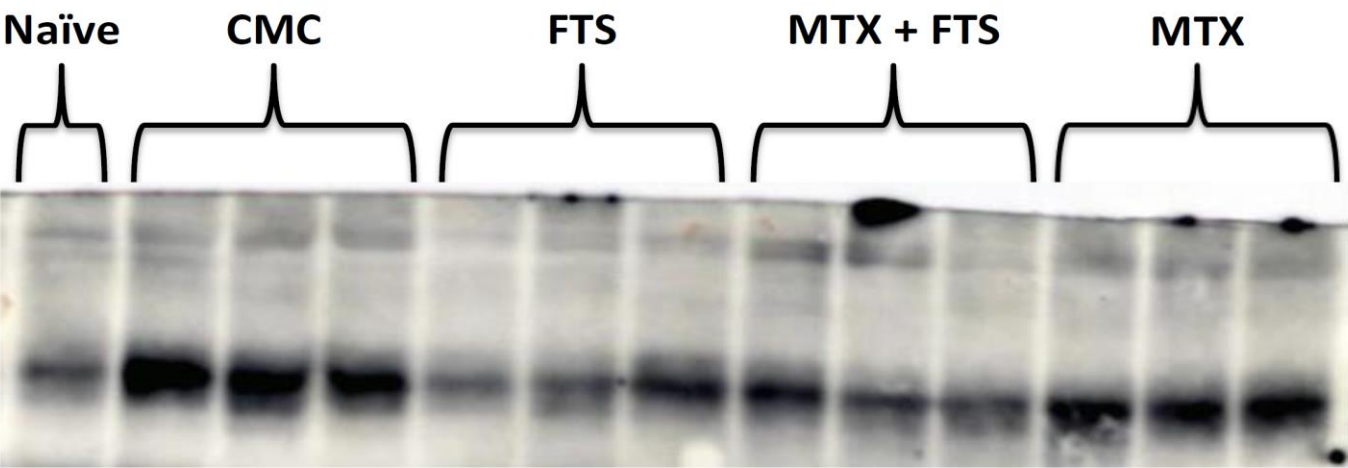


Figure 8

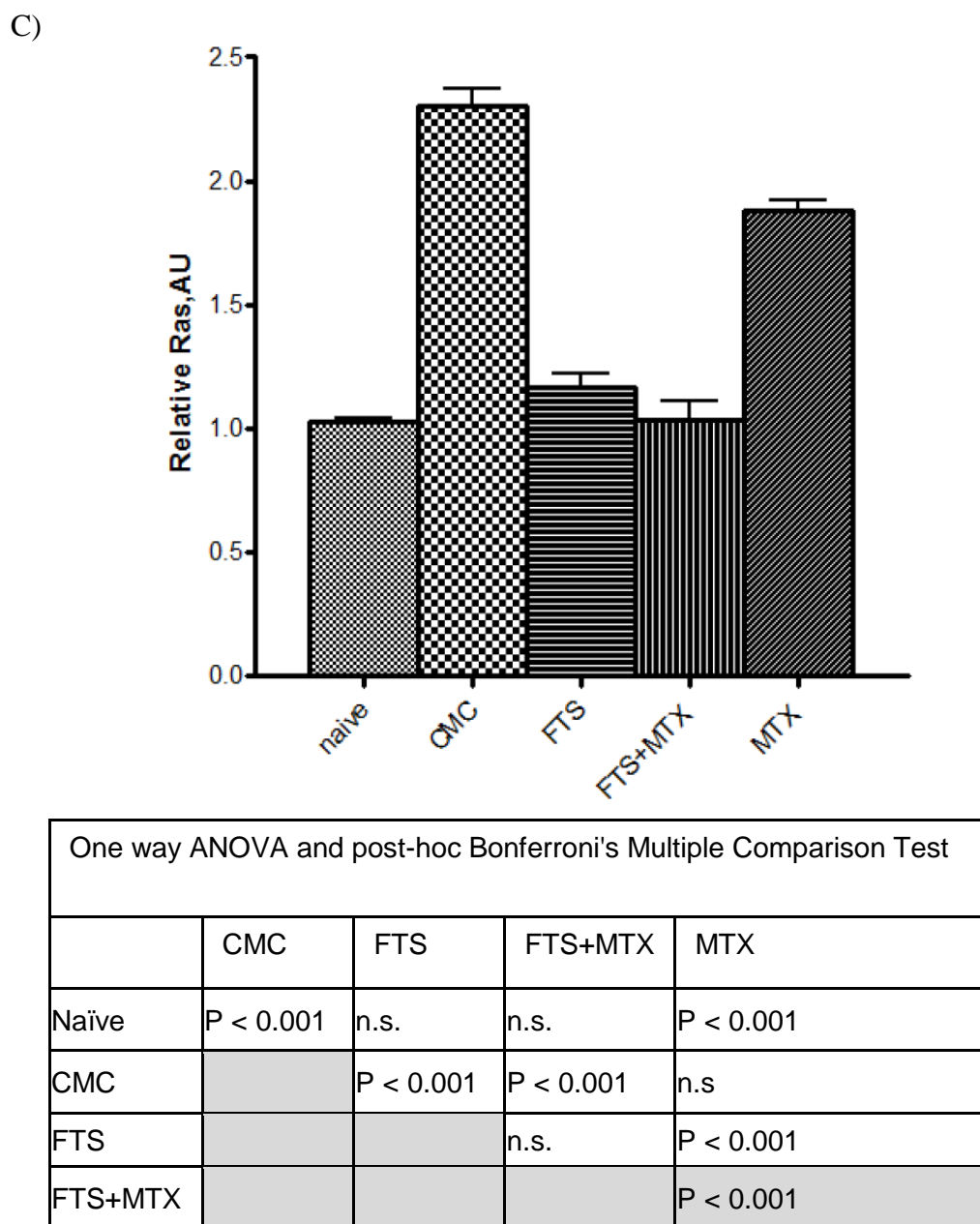


Figure 8. FTS inhibits Ras signaling *in vivo* and total Ras spleen levels during CIA progression. Mice immunized with CFA/CII were treated with FTS (100mg/kg) or CMC (vehicle) starting at day +1 post CIA induction. (A) Single cell suspensions of spleen samples (harvested on day +14 post disease induction) were analyzed by Phospho Specific Flow Cytometry for intracellular phospho-Erk (pERK) and phospho-AKT (p-AKT) levels at the single cell level (Red line CMC, blue line FTS, and green line depicts background staining). (B) In parallel, protein extracts of counterpart spleen cell lysates were analyzed by western blotting (WB) for Ras levels. (C) Depicts the results of the densitometry of a typical experiments done in triplicates. The levels of Ras were normalized to tubulin levels and the bars represent mean \pm SEM of triplicates (arbitrary units; A.U). Statistical analysis between the groups of the results was performed using *One way ANOVA and post-hoc Bonferroni's Multiple Comparison Test* as summarized in the table inset (**P<0.01 by t-test for FTS treatment compared to CMC control treatment).

Figure 9

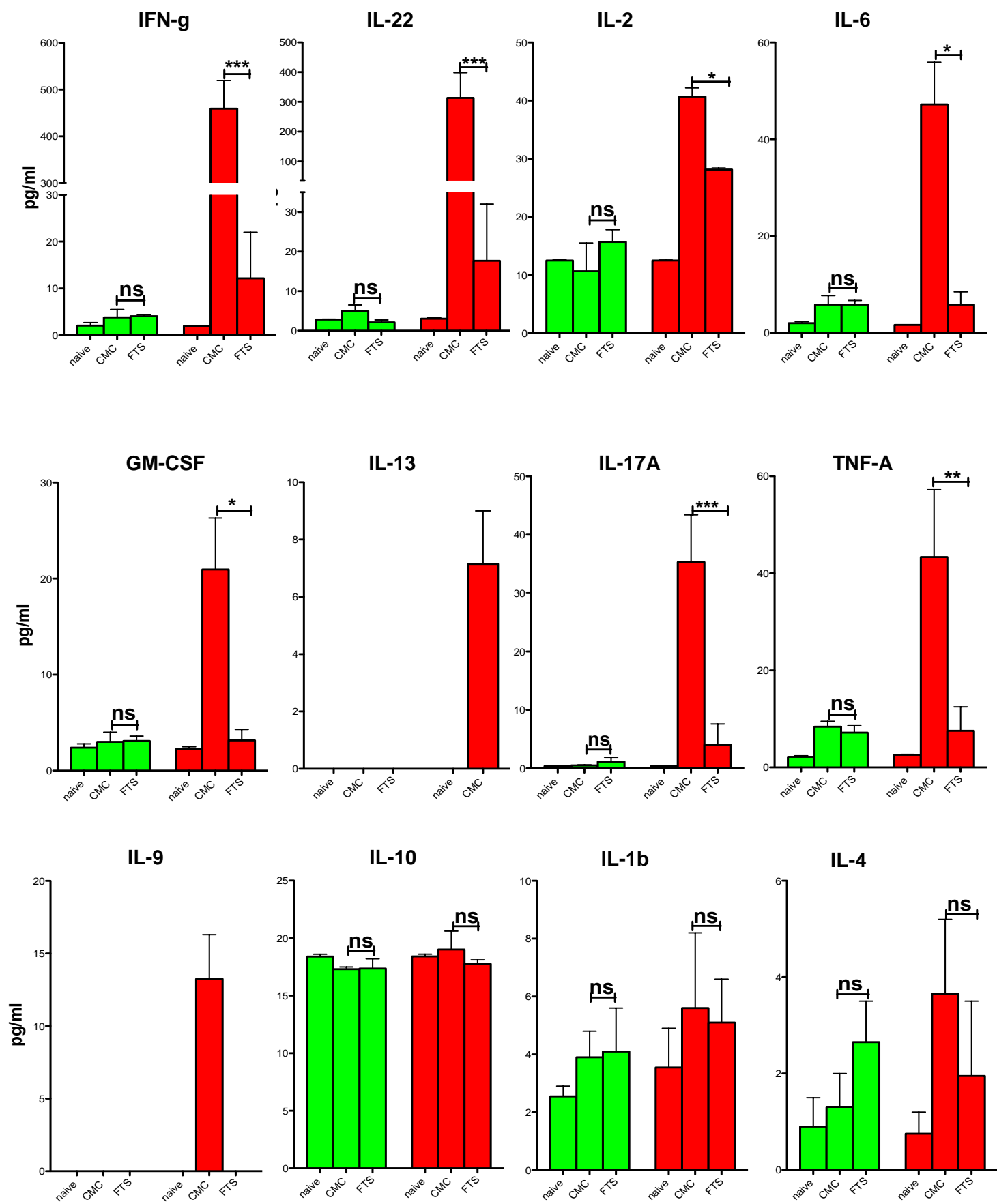


Figure 9. FTS therapy is associated with significant down modulation of TH17-, TH9- and TH1-related cytokine response to CII immunization. We first prepared single cell suspensions from relevant spleen and lymph node samples of mice immunized with CII and treated from Day +1 of the study with oral FTS or CMC vehicle. The cells were immediately stimulated with heat denatured bovine CII (from Chondrex Inc.) or control medium and then culture for additional 72 hours. At the end of culture, the supernatants were collected and we determined the levels of a large number of important cytokines by the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel. Bars represent mean \pm S.E.M of triplicates from a representative experiment, and red bars represent CII stimulated cultures while green bars represent medium control cultures. Data were analyzed for statistical significance t-test for FTS treatment compared to CMC control treatment. Values of $P < 0.001$, $P < 0.01$, and $P < 0.05$ were marked by three, two and one asterisks, respectively.